UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Potential Role of Hexokinase 3 in Neutrophil Development

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by

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The thesis of Mai Iris Mashimo is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2016

DEDICATION

I dedicate this thesis to my family:
my mother, for staying up late with me building a model of the cell in 5th grade, and her relentless support in all my pursuits
my father, for never giving up on his Hollywood dream, and his encouragement for my own dreams
and my brother Raymond, for his existence

I also dedicate this thesis to my other family: Cindy, Nidhi, and Celyn.

TABLE OF CONTENTS

DEDICATION iv

TABLE OF CONTENTS v

LIST OF FIGURES AND TABLES vi

ACKNOWLEDGEMENTS vii

ABSTRACT OF THE THESIS viii

INTRODUCTION 1

MATERIALS AND METHODS 9

RESULTS 14

DISCUSSION 35

REFERENCES 42

SUPPLEMENTARY MATERIAL 45

LIST OF FIGURES AND TABLES

Figure 1. Increase of HK3, but not HK1 and HK2, mRNA levels after G-CSF-mediated neutrophil differentiation of CB-derived myeloid progenitor cells. 25

Figure 2. Increase of HK3, but not HK1 and HK2, mRNA levels after M-CSF-mediated monocyte/macrophage differentiation of CB-derived myeloid progenitor cells. 26

Figure 3. Increase of HK3, but not HK1 and HK2, mRNA levels during G-CSF-mediated neutrophil differentiation of MPB-derived myeloid progenitor cells. 27

Figure 4. HK1, HK2, and HK3 expression in neutrophils and monocytes/macrophages isolated from peripheral blood. 28

Figure 5. The NB4 and HL-60 cell lines differentiated into neutrophil-like cells by ATRA treatment have elevated expression of HK3, but not HK1 and HK2. 29

Figure 6. HK1, HK2, and HK3 knockdown NB4 cell lines. 30

Figure 7. HK1, HK2, and HK3 knockdown HL-60 cell lines. 31

Figure 8. Assessment of HK3 gene disruption in NB4 cells utilizing the T7E1 assay. ... 32

Figure 9. Assessment of NB4 cells lines expressing Cas9/gRNAs targeting HK3. 33

Figure 10. T7E1 assay of expressing Cas9/gRNAs targeting HK3 single clones isolated from cell lines. 34

Table 1. Granulocyte differentiation markers …….……2

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ABSTRACT OF THE THESIS

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Hexokinase (HK) 1, 2, and 3 are metabolic enzymes that catalyze the first step of glycolysis. Previous studies have found that HK3 is directly activated by the transcription factor PU.1 and may be involved in neutrophil differentiation. To examine the role of HK3 in myelopoiesis, the gene expression patterns of HK1, HK2, and HK3 during neutrophil and macrophage differentiation from $CD34^+$ hematopoietic stem and progenitors cells (HSPCs) were investigated. Neutrophil differentiation of myeloid progenitors from cord blood (CB) as well as mobilized peripheral blood HSPCs showed

an increase in HK3 gene expression, while HK1 and HK2 transcripts remained constant. Monocytes/macrophages differentiated from CB HSPCs also showed an increased expression of HK3 mRNA, whereas HK1 and HK2 transcripts were moderately expressed and remained constant. In order to investigate the role of HKs in myelopoiesis, human NB4 and HL-60 promyelocyte cell lines, which demonstrated similar patterns of HK1, HK2 and HK3 gene expression during ATRA-mediated neutrophil differentiation, were utilized to generate shRNA-mediated knockdowns of HK mRNAs. The generated HK shRNA cell lines showed marginal to low decreases in HK levels and also were inconclusive in regards to the extent of neutrophil differentiation. Therefore, I performed CRISPR-Cas9 gene editing of HK3 in NB4 cells. The generated gene-edited cell line had a heterogeneous population and therefore will require additional clonal selection for future experiments. Taken together, my results indicate that there is a prominent increase in HK3 gene expression during myeloid differentiation, suggesting that HK3 may play a role in myelopoiesis and/or neutrophil function.

INTRODUCTION

Hematopoiesis

Hematopoiesis is the process of how blood cells are formed during the lifetime of an individual. The various types of blood cells all arise from hematopoietic stem cells (HSC), originating from the bone marrow (BM). HSCs can differentiate into the myeloid or lymphoid lineages, which in turn give rise to more committed lineages. The myeloid lineage gives rise to common myeloid progenitors (CMPs) that can further differentiate to megakaryocyte/erythrocyte progenitors, which develop to platelets or erythrocytes, or the granulocyte/monocyte progenitors (GMP), also known as myeloblasts, which develop to basophils, eosinophils, monocytes, and neutrophils[1]. Monocytes can further differentiate into macrophages. Extensive research has studied how hematopoiesis is regulated by a network of transcription factors, microRNAs, and cytokines to co-regulate lineage-specific gene expression to direct differentiation.

Promyelocytic cell lines, such as NB4 and HL-60 cells, are cell models to study human myelopoiesis, or myeloid differentiation[2,3]. The promyelocytic cell lines, derived from acute promyelocytic leukemia (AML) patients, have a blockade in cell maturation that prevents further development[2,3]. NB4 cells can be induced to overcome the blockade and differentiate by all-trans retinoic acid (ATRA) treatment[2]. HL-60 cells can be induced to differentiate into neutrophil-like cells by ATRA, dimethylsulfoxide (DMSO), and actinomycin D, or into monocyte/macrophage-like cells by 1,25-dihydroxy-vitamin D_3, phorbol esters, and sodium butyrate[3]. Upon ATRA treatment of the NB4 cell line, the CCAAT/enhancer binding transcription factor ε (CEBPE) is induced and upregulated

through the retinoid acid receptor α (RARα) pathway, leading to differentiation promoting morphological changes towards neutrophil-like cells, such as increased expression of CD11b, CD15, PU.1, and DAPK2.[4] In comparison, monocytic differentiation of primary cells and in the HL-60 cell line does not upregulate CEBPE and DAPK2, but both has upregulated expression of PU.1, CD11b, and CD14[5,6]. Table 1 lists various granulocyte differentiation markers shown to be upregulated in previously published literature. My studies use cell surface markers CD11b, widely used as a myeloid differentiation marker, CD15, a granulocyte differentiation marker and CD14, a monocyte/macrophage marker. [6].

Table 1- Granulocyte differentiation markers

Differentiation Markers	Key characteristics	Expression in myeloid differentiation
PU.1	Transcription factor, regulates myeloid differentiation	↑↑↑ Upon neutrophil differentiation ↑↑↑ Upon macrophage differentiation
CD11b	Myeloid integrin, expressed on mature monocytes, macrophages, granulocytes, NK cells Regulated by PU.1	↑↑↑ Upon neutrophil differentiation ↑↑↑ Upon macrophage differentiation
DAPK2	Death-Associated Protein Kinase 2, tumor suppressor gene, enhancer of neutrophil differentiation Regulated by PU.1	↑↑↑ Upon neutrophil differentiation No change upon macrophage differentiation
CEBPE	CCAAT/enhancer binding protein ε, regulates granulocyte differentiation	↑↑↑ Upon neutrophil differentiation No change upon macrophage differentiation
CD15	Expressed on mature neutrophils, monocytes, promyelocytes	↑↑↑ Upon neutrophil differentiation ↑ Upon macrophage differentiation
CD14	Expressed mainly on macrophages, also expressed on neutrophils	↑ Upon neutrophil differentiation ↑↑↑ Upon macrophage differentiation

PU.1: A Transcription Factor That Is A Master Regulator Of Myeloid Development And Function.

PU.1, an Ets-family member transcription factor, is one of the major transcription factors that regulates myeloid differentiation of CMPs to granulocyte-macrophage progenitors (GMPs) for the development of neutrophils and macrophages[7]. Expression of PU.1 is increased in granulocyte and macrophage lineages and lowered in erythrocyte and megakaryocyte differentiation. PU.1 directly regulates genes involved in myeloid differentiation and function, such as CD11b and DAPK2[8]. CD11b is a key differentiation marker of granulocytes, as it is expressed on mature monocytes, macrophages, granulocytes, and NK cells. PU.1 regulates CD11b expression by binding its promoter during myeloid differentiation[9]. DAPK2, or Death-Associated Protein Kinase 2, is a tumor suppressor gene and is also an enhancer of neutrophil and erythroid differentiation[8,10].

Our lab has previously identified genes regulated by PU.1 via a genetic screen. PU.1-deficient cell lines were generated from PU.1-null mice, and the cells were shown to lack the ability to differentiate into granulocytes or monocytes/macrophages. Microarray analysis was used to compare gene expression between the PU.1-deficient cell line and the PU.1-repleat cell line. The genetic screen found multiple genes dependent on the presence of PU.1, including hexokinase 3 (HK3), a glycolytic enzyme. The regulation of a metabolic enzyme by a transcription factor that regulates myelopoiesis and myeloid function is of considerable interest, and studies may elucidate the potential role of metabolism in myeloid differentiation and function.

Hexokinase Family

Hexokinase (HK) is a glycolytic enzyme that catalyzes phosphorylation of glucose to glucose 6-phosphate in the first step of glycolysis. There are four isoforms of HK. HK1 and 2 are localized in the outer membrane of the mitochondria in mammalian cells. Both are oncogenic when overexpressed and are found in malignant cancer cells [11]. There is a direct correlation between HK1 and 2 activities and tumor progression[12]. HK4 is kinetically and functionally distinct from the other three isoforms, as it plays a role in carbohydrate metabolism in the pancreas and liver. In comparison, HK3 is primarily localized in the cytoplasm of myeloid cells, and exhibits cytoprotective and onco-suppressing effects [11]. HK3 is localized in the cytosol and makes up for 70-80% of the HK activity in granulocytes, with the remaining 20% to 30% of the activity carried out by HK1[13]. Studies have shown that HK3 exerts cytoprotective effects, as overexpression of the enzyme resulted in the decrease of cell death from oxidative stress by increased ATP levels, reduced production of reactive oxygen species, and preservation of the mitochondrial membrane potential[14]. HK activity is pivotal for energy metabolism through the glycolytic pathway since it catalyzes the first step of glycolysis, and its activity may play a role in defining the metabolic state of the cell.

Recent studies by the Tschan and Torbett groups have found that the glycolytic enzyme HK3 is involved in myeloid function and the survival of leukemic blast cells in AML[15]. Lower expression of HK3 was observed in AML patient samples, as compared to normal granulocytes. Furthermore, the downregulation of HK3 expression in AML cells was shown to alter neutrophil development when induced to undergo myeloid

differentiation with the addition of ATRA[15]. These findings imply a potential relationship between metabolism and malignant hematopoiesis in leukemia. Moreover, the findings bring up the question as to the regulation and function of HK3 during normal hematopoiesis and specifically during myeloid development.

Immunometabolism

Recent discoveries reveal that cell metabolism also plays a role in the maintenance of HSC and lineage commitment through shifts in metabolite utilization[16]. Immunometabolism is the interrelationship between cellular energetics and the immune response, where metabolic pathways regulate differentiation, function, and survival of immune cells. There are two main pathways for energy metabolism to generate ATP for energy: glycolysis and oxidative phosphorylation. Glycolysis is the glycolytic metabolic pathway that breaks down glucose to yield ATP, pyruvate, and other metabolites and occurs in the cytosol. HK catalyzes the first step in glycolysis, in phosphorylation of glucose to glucose-6-phosphate. Oxidative phosphorylation is the process of phosphorylation of ADP into ATP by utilization of electrons from NADH and $FADH_2$ with oxygen, and takes place in the mitochondria. Metabolic preferences arise from the cellular environment and cellular energetic demands specific to different cell types. HSCs reside in the bone marrow, which is a hypoxic niche, or a low-oxygen tension environment. In hypoxic conditions, anaerobic glycolysis is the favored method of glucose metabolism, and this metabolic state plays a role in maintaining the self-renewal function in HSCs[17]. The preference of glycolysis over normal mitochondrial respiration also protects HSCs from reactive oxygen species production, which can induce DNA

damage. Recent studies have found how different metabolic pathways contribute to hematopoiesis. For example, the commitment to the erythroid lineage is found to be dependent on glutamine transport and metabolism, where the absence of glutamine resulted in a shift to the myelomonocytic cell lineage[18]. This report also demonstrated the metabolic preference of glutamine catabolism over glycolysis in erythroid differentiation by finding increased erythroid commitment in HSCs when treated with 2-deoxyglucose (2-DG), an inhibitor of HK[18]. The metabolic preference of the myeloid cell lineage is not yet clearly elucidated, but the study in erythroid lineage metabolism suggests that neutrophil and monocyte/macrophage differentiation is dependent on glycolysis.

Although glycolysis is not the most efficient metabolic pathway to generate ATP, it plays a major role in the activation and function of myelomonocytic cells, macrophages, dendritic cells, and neutrophils[19]. Macrophages and dendritic cells are two key players of the innate immune response that are on the frontline of defense that are derived from monocytes. Innate cells recognize foreign pathogens through pattern recognition receptors (PRR), that when activated, result in the pro-inflammatory immune response. The activation of the PRR not only results in an activation of the signaling cascade to increase expression of inflammation related genes, but also induces a metabolic reprogramming of the now activated immune cell to undergo increased levels of glycolysis[16,19]. Treatment with 2-DG, a HK inhibitor of all HK isoenzymes, was found to decrease the activation of macrophages and inflammatory responses, while inhibition of oxidative metabolism had no effect[19]. Metabolism also plays a role in directing different macrophage function depending on the stimulus used, as activation of

macrophages with LPS results in increased glycolytic activity, while activation by Interleukin-4 results in the utilization of the Krebs cycle over glycolysis[19]. Neutrophils, a member of the myelomonocytic cell lineage, also heavily utilize glycolysis as the primary energy source. Neutrophils have little to no dependence on oxidative phosphorylation, as they contain very few mitochondria, which they utilize for apoptotic caspase activation rather than for metabolism[20].

Although the role of immunometabolism in the function of immune cells has been studied, regulation of metabolism in the context of hematopoiesis and myeloid differentiation remains unclear. This link between the myeloid master regulator transcription factor PU.1 and the glycolytic enzyme HK3 suggests that metabolic pathways function in tandem with transcriptional and translational regulatory mechanisms in the development of neutrophils and macrophages.

Investigation of Hexokinase in Neutrophil and Macrophage Differentiation

Given that glycolysis is the major metabolic pathway in neutrophils and macrophages and plays a role in immune cell function, and that HK3 is a target of myeloid transcription factor PU.1, it is of interest to study the glycolytic enzyme in myeloid differentiation. Studying the role of HK in neutrophil and macrophage differentiation would elucidate the glycolytic enzyme's relationship to myelopoiesis as well as the three isozyme's role in myeloid differentiation and function and the relationship between the three isozymes during these processes. This would provide some insight into the possible role of metabolism in normal myelopoiesis and in disorders that arise from dysfunction in myelopoiesis, such as AML and neutropenia. My goals were to 1) examine gene

expression patterns of HK 1, 2, and 3 in human primary hematopoietic cells during differentiation of hematopoietic stem and progenitor cells (HSPCs) to neutrophils and monocytes and 2) investigate the biological relevance of HK 1, 2, and 3 in neutrophil differentiation by generating knockdown and knockout cell lines.

MATERIALS AND METHODS

Chemicals and reagents. Stem cell factor (SCF), interleukin 3 (IL-3), interleukin 6 (IL-6), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF) were obtained from PeproTech. Dulbecco's modified Eagle's medium (DMEM), Iscove's Modified Dulbecco's Medium (IMDM), Roswell Park Memorial Institute medium (RPMI-1640), L-glutamine and penicillin/streptomycin were obtained from ThermoFischer Scientific. Fetal bovine serum (FBS) was obtained from GE Healthcare Life Sciences. All-trans retinoic acid (ATRA) was purchased from Sigma-Aldrich.

NB4 and HL-60 cell culture. The human APL cell lines NB4 and HL-60 were maintained in RPMI-1640 supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin. The human embryonic kidney *293T* cells were cultured in DMEM supplemented with 5% FBS, 1% penicillin/streptomycin. The cells were maintained in a humidified 5% CO_2 atmosphere at 37^{o}C. Cells were counted using a hemocytometer chamber, and viability was determined by trypan blue dye exclusion. Cells were passaged when the cells reach a maximum cell density of 1E6 cells/ml and split to 0.1-0.2E6 cells/ml every 2-3 days.

Primary cell culture. Frozen mobilized $CD34^{+}$ cells isolated from a healthy donor (provided by City of Hope). Cells were expanded for 9 days in IMDM medium supplemented with 10% FBS, 100ng/ml SCF, 50ng/ml IL-3, 50ng/ml IL-6, and 1% penicillin/streptomycin at a cell density of 1E6 cells/ml. The cells were maintained in a humidified 5% CO_2 atmosphere at 37^{o}C. The cells were differentiated into neutrophils in

IMDM medium supplemented with 0.1μg/μl G-CSF, 0.1μg/μl IL-6, 10% FBS, and 1% penicillin/streptomycin over a period of 12 days.

Human cord blood from two donors was obtained from the Cleveland Cord Blood Center. Human cord blood $CD34^+$ cells, under approved institutional protocol and in accordance with the Declaration of Helsinki, were isolated using the EasySep Human Cord Blood CD34 Positive Selection Kit in accordance with the manufacturer's instructions (STEMCELL Technologies). $CD34^+$ cells were expanded in IMDM supplemented with 10% FBS, 100ng/ml SCF, 50ng/ml IL-3, 50ng/ml IL-6, and 1% penicillin and streptomycin at a cell density of 1E6 cells/ml. Cells were either differentiated into neutrophils in IMDM medium with 0.1μg/μl G-CSF, 0.1μg/μl IL-6, 10% FBS, and 1% penicillin/streptomycin, or into macrophages in RPMI-1640 medium with 50ng/ml G-CSF, 20ng/ml M-CSF, 10% FBS, and 1% penicillin/streptomycin. Cells chosen for macrophage differentiation were then switched to RPMI-1640 without GM-CSF after three days of culture. Cells were maintained at 1E6 cells/ml over the duration of differentiation.

After approval from The Scripps Research Institute (TSRI) Institutional Review Board, La Jolla, CA, USA, human blood was obtained from healthy donors from the Normal Blood Donation Center at TSRI. Monocytes were isolated from two donors using the RosetteSep Negative Selection kit (Stem Cell Technologies). Isolated cells were cultured for 6 days in RPMI-1640 containing 10% FBS, 1% L-Glutamine, 1% penicillin/streptomycin, and 20ng/ml of M-CSF.

Neutrophils isolated by negative selection from human blood were provided by Dr. Keri Mowen (The Scripps Research Institute, La Jolla, CA).

Lentiviral vector production. pLKO.1-puro lentiviral vectors expressing small hairpin RNA (shRNA) targeting HK1 (shHK1_2050: NM_033498.1-2050s1c1/TRCN0000037658), HK2 (shHK2_2933: NM_000189.4-2933s1c1/TRCN0000195582), and HK3 (shHK3_1420: NM_002115.1-1420s1c1/ TRCN0000037679) and a non-target shRNA Control (SHC002) were purchased from Sigma-Aldrich. All vectors contain a puromycin antibiotic resistance gene for selection of transduced mammalian cells. The lentiCRISPRv2 vector containing Cas9 and a puromycin selection marker was purchased from (Zhang Lab, Addgene). Lentiviral vectors for shRNA knockdown and CRISPR knockouts were generated by transient transfection of the corresponding plasmid and third-generation packaging plasmids pMD.G (VSV-G), pMDLg/p.RRE (gag and pol), and pRSV-Rev (rev). The 293T cells were transfected using Polyethylenimine (PEI), and the supernatant was collected after 24 and 48 hours post-transfection. The media was filtered through a 0.45µm nitrocellulose membrane and concentrated by ultracentrifugation at 19,400 rpm for 2 hours and 20 minutes at 4ºC in a Beckman SW28 rotor and resuspended in RPMI-1640 medium.

Generation of HK1, HK2, HK3 Knockdown cell lines. Lentiviral vectors were used to transduce NB4 and HL-60 cells by spinoculation. 0.125E6 NB4 or HL-60 cells were incubated with 2-50µl of the concentrated virus and 8µg/ml polybrene for 30 minutes in a humidified incubator at 37ºC, then centrifuged at 1000g for 90 minutes at 37ºC. Infected cells were resuspended in RPMI-1640 supplemented with 20% FBS, 1% L-glutamine and 1% penicillin/streptomycin. After 2-7 days post-spinoculation, cells were selected by the addition of 1.5µg/ml puromycin.

Generation of HK3 gene-edited cell lines using CRISPR. Three different HK3 knockout cell lines were generated using different guide RNAs targeting HK3 (Table S1). NB4 cells were transduced by spinoculation and cells were selected by 1.5μg/ml puromycin 4 days post-transduction (as previously described).

T7 Endonuclease 1 Assay. Genomic DNA of the region spanning the target sequence was amplified by PCR and purified using the MinElute PCR Purification Kit (Qiagen). For T7E1 cleavage assay, purified PCR products were denatured and annealed in NEBuffer 2 (NEB) using a thermocycler. Hybridized PCR products were digested with T7 endonuclease 1 (NEB, M0302L) for 15 minutes at 37°C and subjected to 1% agarose gel electrophoresis.

ATRA Differentiation. NB4 or HL-60 cells at a density of 0.1E6 cells/ml were induced for granulocytic differentiation by addition of 5μM ATRA in a 12 well plate. Cells were maintained below 1E6 cells/ml over 6 days, and fresh ATRA was added at day 3.

Cytospins. Microscope slides, cytospin chambers, and filter cards were prepared for each cytospin slide. Cell fractions (approximately 0.05E6 cells/ml) were suspended in 1X phosphor-buffered saline (PBS) supplemented with 20% FBS in 100μl. Centrifugation for 5 minutes at 450 rpm was performed using the Shandon Cytospin 2 centrifuge. Cells were fixed for one minute by several drops of methanol. The slides were then stained for visualization with the Wright-Giemsa stain for approximately 30 seconds, washed with ultrapure Milli-Q water, and air-dried.

Real-time quantitative PCR. RNA was isolated using the RNeasy Mini Kit (Qiagen), and complementary DNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). The expression of mRNA of HK1, HK2, HK3, DAPK2, PU.1, and CEBPE was assayed

using the TaqMan Gene Expression Assay. The reactions were run on the Roche LightCycler 480 II (Roche Life Science). The qPCR reactions contained 2.5 µl of cDNA (equivalent to 50 ng of total RNA), 10 µl of 2X LightCycler 480 Probes Master (Roche) and 2µl of 10X GAPDH primer or 1µl of the other 20X primers in 20 µl total volume. The primers for qRT-PCR used were HK1 Hs00175976_m1-44553320, HK2 Hs00606086_m1-44553320, HK3 Hs01092839_m1, PU.1 Hs00231368_m1, DAPK2 Hs00204888_m1, CEBPE Hs00357657_m1, GAPDH Hs.PT.39a.22214836 (ThermoFischer Scientific). The cycling conditions were at 95°C for 10 min pre-incubation, followed by amplification by 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C, then a cooling step of 37°C for 30 seconds. Each sample was run in technical duplicates. Comparative threshold values were acquired using LightCycler 480 Software Version 1.5. The relative N-fold change in expression was calculated using the equation $2^{-\Delta\Delta Ct}$.

Flow cytometry. Cells were assessed for differentiation by flow cytometry analysis of surface markers of granulocytic or monocytic maturation using anti-human CD11b, CD15, and CD14 antibodies conjugated with appropriate fluorophores (BD Biosciences). 0.5E6 cells in 50µl of ice-cold fluorescence-activated cell sorter buffer (5mM EDTA, Gibco and 1% BSA, Bioworld in DPBS, Corning) were stained with 2-3µl of each antibody at 4°C for 20 minutes in the dark, washed, and analyzed using the BD LSR II equipped with the BD FACSDiva software. Data analysis was done using FlowJo software.

RESULTS

1. Expression of hexokinase isozymes during myelopoiesis in neonatal and adult human hematopoietic stem and progenitor cells

1.1. Hexokinase 1, 2, and 3 mRNA gene expression patterns in neonatal umbilical cord blood hematopoietic stem and progenitor cells during myelopoiesis and in neutrophils and monocytes/macrophages.

In order to investigate HK mRNA expression patterns and levels in myeloid cells after differentiation, $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) isolated from umbilical cord blood (CB) were differentiated to neutrophils by the addition of Granulocyte-Colony Stimulating Factor (G-CSF), or differentiated to monocyte/macrophages by the addition of Macrophage-Colony Stimulating Factor (M-CSF). Neutrophils and monocytes/macrophages arise from a common myeloid progenitor during HSPC differentiation. To determine whether HK gene regulation patterns are unique to neutrophil differentiation or common among both neutrophils and monocytes/macrophages, patterns and levels of HK mRNA expression levels were determined. Successful differentiation to either lineage was confirmed by lineage-specific morphological changes, surface expression of differentiation markers, and mRNA expression levels of genes involved in differentiation with either cell lineage. Neutrophil differentiation by G-CSF by 6 days resulted in segmented neutrophils (Figure 1A). There was a 3-4 fold increase in the surface expression of the myeloid differentiation marker CD11b and neutrophil differentiation marker CD15 (Figure 1B). CD14, also expressed

on monocytes and macrophages, had increased expression levels (Figure 1B). Correspondingly, there was approximately a 2-fold increase in expression of CEBPE, PU.1, and DAPK2 (Figure 1C). Neutrophil differentiation resulted in a significant 14-fold increase in HK3 mRNA expression (Figure 1D). HK1 and HK2 mRNA expression was present and remained constant before and after differentiation. The significant increase in HK3 gene expression over the course of neutrophil development in HSPCs suggests that HK3 may play a role in neutrophil differentiation and/or later function.

To determine HK mRNA expression patterns and levels in monocytes/macrophages, progenitors were treated with M-CSF and 6 days post treatment, large, vacuolated, adherent cells were observed indicating that macrophage differentiation resulted (Figure 2A). The significant increase in cell surface expression of CD11b and CD14 further confirmed macrophage differentiation (Figure 2B). Correspondingly, mRNA expression levels of CEBPE increased 3-fold (Figure 2C), with moderate increases of CD15 and low increases of DAPK2 mRNA expression levels, as compared to neutrophils, which was expected. During macrophage differentiation, HK1 and HK2 expression remained constant, similar to trends observed in neutrophils (Figure 2D). In contrast, HK3 expression was increased 4-fold in macrophages. Although neutrophils had a 14-fold increase in HK3 expression, the elevated level of HK3 in macrophages after development suggests that HK3 may play a role in macrophage development/function as well.

1.2. Hexokinase 1, 2, and 3 mRNA gene expression patterns in adult mobilized peripheral blood hematopoietic stem and progenitor cells during myelopoiesis and in neutrophils.

The findings from neonatal HSPCs indicated that HK3, but not HK1 and 2, increased during myelopoiesis. I then investigated gene expression of HK1, 2 and 3 during neutrophil development in $CD34^+$ HSPCs isolated from mobilized peripheral blood (MPB) that were differentiated to neutrophils by the addition of G-CSF. Neutrophil differentiation was confirmed by morphological changes, surface expression of differentiation markers, and differentiation marker gene expression over 12 days. mRNA was collected at selected time points. Changes in HSPC morphology were observed as differentiation progressed and cells developed a multi-lobed nucleus, a characteristic of neutrophils (Figure 3A). Surface expression of the myeloid differentiation marker CD11b and neutrophil differentiation marker CD15 increased with progressing stages of G-CSF-induced differentiation (Figure 3B). CD14, which is primarily a monocyte and macrophage marker, was moderately expressed on neutrophils. Increased mRNA expression levels of CEBPE, PU.1, and DAPK2 were also observed throughout neutrophil development (Figure 3C). During the progression of neutrophil differentiation from HSPCs the expression of HK1 and HK2 remained constant, whereas the expression of HK3 significantly increased (Figure 3D). HK3 expression steadily increased during the 12-day period of neutrophil differentiation resulting in a 4-fold increase of HK3 relative to undifferentiated HSPCs. The significant increase of HK3 mRNA expression, but not HK1 and HK2 mRNA expression, was observed in myeloid progenitors and neutrophils derived from both CB and MPB, suggesting HK3 regulation is independent of

ontological age and may play an important role in neutrophil differentiation and/or function.

1.3. Hexokinase 1, 2, and 3 mRNA gene expression patterns in monocytes/macrophages and neutrophils isolated from peripheral blood.

In addition to the studies with $CD34^+$ HSPCs isolated from MPB and CB, mature neutrophils and macrophages were isolated from peripheral blood in order to investigate the levels of HK expression in primary cells obtained directly from their *in situ* environment. Initially, HMBS (hydroxymethylbilane synthase) was used as housekeeping, control gene for mRNA expression analysis, but was found to not be an ideal reference gene, as it had a high cycle threshold (Ct) value of 28.88 in the undifferentiated MPB $CD34^+$ HSPCs obtained from peripheral blood, and was determined to be inappropriate when it was not expressed at detectable levels in neutrophils (Ct=35). Furthermore, and unfortunately, neutrophil RNA samples were limited due to very low RNA content (0.1μg of total RNA in 10^6 cells); therefore, Ct values were directly compared, under the assumption of constant input of cDNA. The Ct value is the number of cycles required for the fluorescent signal to cross the threshold of background fluorescence; the Ct values are inversely proportional to the amount of target nucleic acid in the sample.

Neutrophils had an average Ct value of 30.81 for HK1, 30.77 for HK2, and 28.37 for HK3 (Figure 4A). Assuming equivalent cDNA input, the lower Ct value of HK3 indicates higher HK3 mRNA levels in neutrophils as compared to HK1 and HK2 mRNA levels. The Ct value of HK3 is smaller than HK1 and HK2 by 2.42, indicating a 5.35-fold higher mRNA expression of HK3 than either the HK1 and HK2 isoforms.

Macrophages had an absolute Ct value of 25.06 for HK1 mRNA, whereas, the Ct value was 23.04 for HK2, and 20.89 for HK3 (Figure 4B). The Ct value was lowest for HK3, indicating higher HK3 mRNA levels in macrophages, while HK1 and HK2 mRNAs were only moderately expressed. In addition to the *in vitro* differentiation studies with HSPCs, primary neutrophils and monocytes/macrophages directly isolated from peripheral blood further show that HK3 is highly expressed.

2. Expression of HK isozymes in the promyelocyte cell lines NB4 and HL-60

2.1. HK gene expression during differentiation of the promyelocyte cell line NB4

It has been previously demonstrated that HK3 transcripts, but not HK1 and HK2 transcripts, were induced during ATRA-induced neutrophil differentiation of the promyelocyte cell line NB4[15]. I confirmed HK 1, 2, and 3 transcript expression patterns and levels in NB4 cells treated with 5μM of ATRA for 6 days. ATRA differentiated cells exhibited the neutrophil characteristic horseshoe shaped nucleus and granules (Figure 5A). The surface expression of differentiation marker CD11b showed an increase by over 10-fold, as found similarly in other studies with ATRA differentiation of NB4 cells[15] (Figure 5B). The neutrophil markers CD15 and CD14 do not have an as significant increase as CD11b, with only a 1.5-2 fold difference between undifferentiated NB4 to ATRA-treated cells (Figure 5B). Neutrophil-like differentiation was also confirmed by CEBPE, PU.1, and DAPK2 transcript expression (Figure 5C). HK1 and HK2 transcript expressions were evident, but not elevated during differentiation (Figure 5D). As reported previously by our group, HK3 transcript expression increased; I determined a 900-fold

HK3 transcript induction as compared to untreated cells (Figure 5D). The NB4 cell line is thus an effective model cell line for investigating the role of hexokinases in neutrophil differentiation.

2.2. HK gene expression during differentiation of the promyelocyte cell line HL-60

In addition to the characterizing HK transcript expression in NB4 cells, I evaluated HK transcript expression in the promyelocyte HL-60 cell line. Similar to the NB4 cells, HL-60 cells were treated with 5μM of ATRA for 6 days to promote neutrophil differentiation. HL-60 cells displayed a horseshoe shaped nucleus, but were not as granular or "foamy" as differentiated NB4 cells (Figure 5E). The expression levels of the differentiation markers CD11b, CD15, and CD14 were similar to NB4 cells, with CD11b showing a 14-fold increase after differentiation (Figure 5F). CD15 and CD14 transcripts increased slightly, 1.5-2-fold (Figure 5F). Interestingly, mRNA expression of CEBPE and PU.1 decreased after ATRA differentiation, while DAPK2 transcripts demonstrated a 28-fold increase (Figure 5G). Although ATRA-induced differentiation in HL-60s may not be as robust as in NB4 cells, the hexokinase expression of ATRA-treated HL-60s exhibit similar trends in HK1, HK2, and HK3 expression patterns; HK3 exhibited 10-fold increase in transcript expression after differentiation, whereas HK1 and HK2 remained constant (Figure 5H). My analyses of HK1, 2, and 3 transcript expression patterns in primary cells and cell lines demonstrated similar trends during differentiation of myeloid progenitors to neutrophils, HK3 was upregulated, whereas HK1 and HK2 transcript expression remained fairly unchanged.

3. Knockdown of HK1, HK2, and HK3 transcripts in NB4 and HL-60 cells using lentiviral vector delivered shRNA

3.1. The Consequence of Knocking Down HK Transcripts in NB4 Cells

To investigate the role of the three hexokinase isozymes in myeloid differentiation, knockdowns of HK1, HK2, and HK3 were undertaken in the promyelocyte cell lines NB4 and HL-60 using lentiviral vector delivered shRNA. NB4 and HL-60 cells were established utilizing shRNAs targeting each of the three hexokinase mRNAs or with a "scrambled" non-targeting shRNA (SHC002) to serve as a control. Given that HK3 is upregulated during ATRA differentiation, I evaluated the efficiency of the generated knockdowns after treating the cell lines with ATRA for 6 days. In the NB4 cell line, HK1 knockdown demonstrated the most significant decrease of HK1 transcript expression, with a decrease of 90% as compared to the scrambled control (Figure 6A). ATRA-induced differentiation of the hexokinase knockdowns was further assessed by surface expression of the differentiation markers CD11b, CD14, and CD15 and by mRNA expression of CEBPE, PU.1, and DAPK2. The HK1 knockdown resulted in less differentiation, as there was a 20% decrease in CD11b, 13% decrease in CEBPE, 50% decrease in DAPK2, and 34% decrease in PU.1 expression, in comparison to the shRNA control line (Figure 6A, B). There was no change in expression of the neutrophil marker CD15, nor the monocyte/macrophage marker CD14. Interestingly, in the HK1 shRNA, the transcript expression of HK2 and HK3 were also significantly reduced by over 50% (Figure 6A). The change in expression levels of myeloid genes from disruption in HK transcript may suggest that HKs play a role in neutrophil differentiation. Unfortunately,

the HK2 and HK3 shRNA cell line in NB4 cells demonstrated minimal mRNA expression, with a decrease of 25% and 26%, respectively (Figure 6D, G). Although the HK2 and HK3 shRNA NB4 cell lines differentiated slightly less than the scrambled control cell line, the level of knockdowns and was insufficient to draw conclusions on the role of the HKs in differentiation.

3.2. The Consequence of Knocking Down HK Transcripts in HL-60 Cells

Hexokinase transcript knockdowns were generated in the HL-60 cell line utilizing the same series of shRNAs as used to generate the NB4 cell lines. The HK1 knockdown had a significant effect on HK1 mRNA expression resulting in a decrease of 72% (Figure 7A). In contrast to the HK1 knockdown NB4 cell line findings, the HK2 and HK3 transcript levels were unchanged in the HK1 knockdown HL-60 cell line (Figure 7A). Similar to the HK1 knockdown NB4 cell line, there was an approximate 50% decrease in PU.1 and DAPK2 transcripts in HL-60 cells. However, CEBPE, CD11b, CD15, and CD14 transcripts showed no change, with 1 to 1.3-fold transcript increase after differentiation (Figure 7A, B). Although the HK1 knockdown NB4 cell line exhibited a decrease in neutrophil differentiation, the alteration of differentiation was not evident in HK1 knockdown HL-60 cell line.

The HK3 knockdown HL-60 cell line showed a 59% reduction of the HK3 transcript, as compared to the scrambled shRNA cell line (Figure 7G). The HK3 knockdown cell line was found to have decreased HK1, 48%, and HK2, 21%, transcript levels (Figure 7G). The HK3 knockdown cell line had an approximate 40% decrease in PU.1 and DAPK2 transcripts, gene programs required for neutrophil development and

function, as compared to the SHC002 control, but a small 1.2- to 1.4-fold increase in CEBPE, CD11b, CD15, and CD14 (Figure 7G, H), usually modulated with neutrophil development[15,3]. Lastly, HK2 transcripts were decreased marginally, 27%, in the HL-60 cell line (Figure 7D). Overall, alteration of common patterns of neutrophil-specific gene expression associated with differentiation was not evident in HK1, HK2, and HK3 hexokinase knockdown HL-60 cell lines. Moreover, evidence of cellular alteration in the various HK knockdown cell lines was inconclusive.

4. Knockout of HK3 in NB4 cells using CRISPR/Cas9

HK1 knockdown in NB4 cells decreased neutrophil differentiation. However, it is not clear whether the lowered expression of HK1 led to decreased neutrophil differentiation, or if this was due to the reduced levels of HK2 and HK3 present in the cell line after knocking down HK1. To start to unravel the role of each hexokinase isozyme in myeloid development, I developed HK3 CRISPR for disrupting HK3 gene expression. A HK3 knockout cell line would help clarify whether my observed results showing partial disruption of HK1 in NB4 cells were due to disrupting HK1 gene expression or a combination of events as the result of decreasing HK2 and HK3 mRNA transcripts and cellular function. Moreover, by disrupting each HK completely, compensatory effects can be investigated when there is a loss of HK function allowing investigation of the regulatory relationships between the three isozymes and resulting cellular function.

Three NB4 cell lines were created, each with a 20-22nt gRNA targeting exons 2, 3 or 4 of HK3, and Cas9. The T7E1 assay showed that there was successful cleavage by Cas9 for gRNA 1 and gRNA 3, but I found that the cell line is a heterogeneous population of single and bi-allelic HK3 gene alterations and wild type cells (Figure 8A). ATRA treatment of the bulk cell lines resulted in a 30%-49% decrease of HK3 mRNA expression, a 2-fold increase in HK1 mRNA expression, and 1.59 to 1.97-fold increase in HK2 mRNA expression in the three cell lines (Figure 9A, D, G). The analysis of HK3 mRNA expression however was not a good indication of the extent of HK3 gene disruption, as the primers used in the RT-PCR reaction might detect modified HK3 mRNA, rather than a disrupted message. Regardless, mRNA expression for HK3 decreased in the three independently derived NB4 cell lines.

The originally reported shRNA HK3 knockdown cell lines, selected for shRNA expression, were impaired in ATRA-induced neutrophil differentiation, as seen by a 50% reduction in CEBPE mRNA and CD11b surface expression. Therefore, it might be anticipated that the NB4 Cas9/gRNAs targeting HK3 cell lines would have altered neutrophil differentiation after ATRA-treatment. However, there was no change in the expression of myeloid markers CEBPE, PU.1, and DAPK2, and no significant change in expression of surface markers CD11b and CD15 (Figure 9). In summary, the NB4 Cas9/gRNAs targeting HK3 cell lines manifested no observed differences upon ATRA-induced neutrophil differentiation. This observation might be anticipated, given that I observed a mixture of gene-edited HK3 single and bi-allelic genes and HK3 wild type alleles in HK3 Cas9/gRNAs targeted NB4 cell lines.

To isolate bi-allelic gene-edited HK3 cells, cells were cloned by limiting dilution assays. Several of the obtained single clones selected by limiting dilution were expanded. Utilizing the T7E1 assay to evaluate four single clones obtained from gRNA1 lines, one line was a mono-allelic for HK3 gene editing, as bands representing the wildtype HK and HK gene edit allele were both present, whereas the other three lines presented with one band, which may represent either a wild type or a bi-allelic HK3 gene edit (Figure 10). The findings showed that the cells are able to grow from limiting dilution with HK gene editing. Furthermore, additional single clones were generated by seeding 1 cell per well in a 96 well plate for each gRNA line. However, given that the results were not conclusive as to HK3 bi-allelic gene editing due to the nature of the assays, studies are underway to utilize sequencing to determine HK3 indels, and determine changes in HK3 protein expression by mass spectrometry to demonstrate HK3 gene disruption.

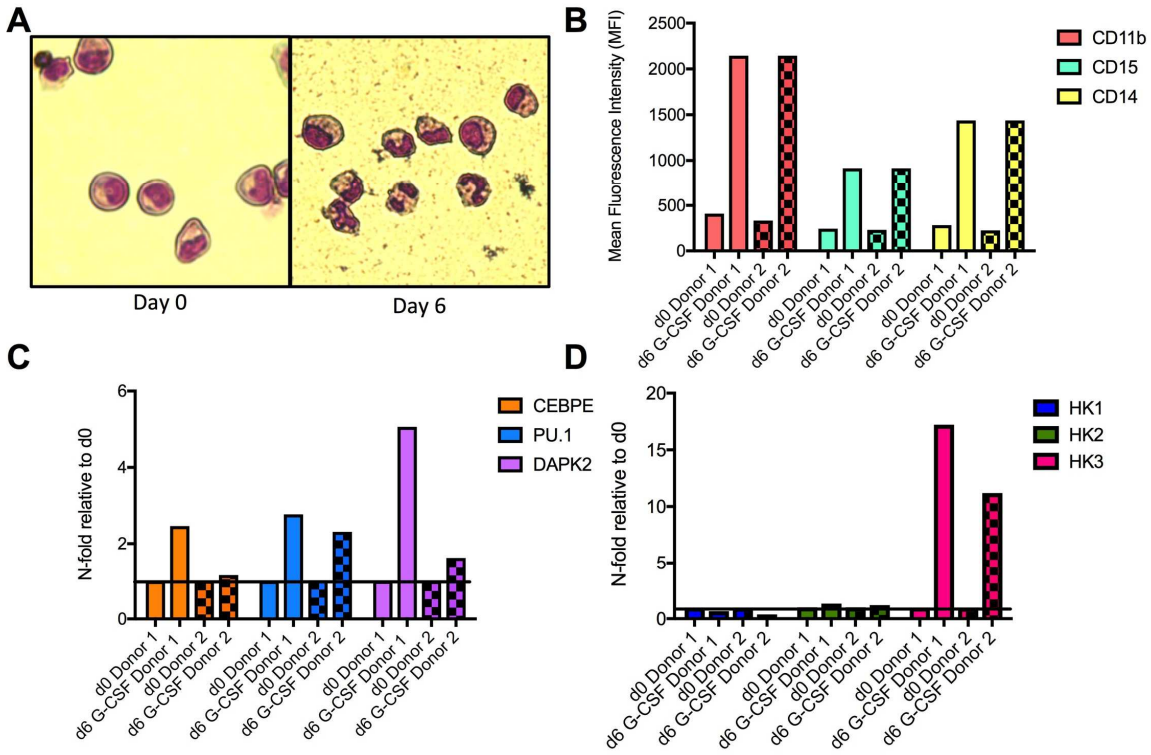

Figure 1. Increase of HK3, but not HK1 and HK2, mRNA levels after G-CSF-mediated neutrophil differentiation of CB-derived myeloid progenitor cells.
(A) Assessment of HSPC neutrophil differentiation by G-CSF treatment. $CD34^+$ HSPCs were obtained and expanded from CB of two donors. The myeloid progenitors were differentiated toward neutrophil-like cells by treatment with G-CSF and morphological changes were assessed after day 6 by Wright-Giemsa staining. (B) Flow cytometric analysis pre- and post-G-CSF treatment. Expression levels of CD11b, CD15, and CD14 after G-CSF treatment of myeloid progenitor cells were evaluated by flow cytometry. (C) Quantification of mRNA expression from selected myeloid genes pre- and post-G-CSF treatment. mRNA expression of CEBPE, PU.1, and DAPK2 after G-CSF treatment of myeloid progenitor cells was determined by RT-PCR. Values are normalized to the housekeeping gene GAPDH, and shown by the N-fold change compared to cells before G-CSF treatment. (D) Quantification of HK1, HK2, and HK3 mRNA expression pre- and post-G-CSF treatment. mRNA expression of HK1, HK2, and HK3 after G-CSF differentiation of $CD34^+$ cells was determined by RT-PCR, and is presented as the N-fold change compared to the pre-G-CSF myeloid progenitors. In B, C and D the data is presented as the mean of the technical replicates of each donor; the technical replicates are within 5% of each other (B, C, D: n=2, 2 technical replicates).

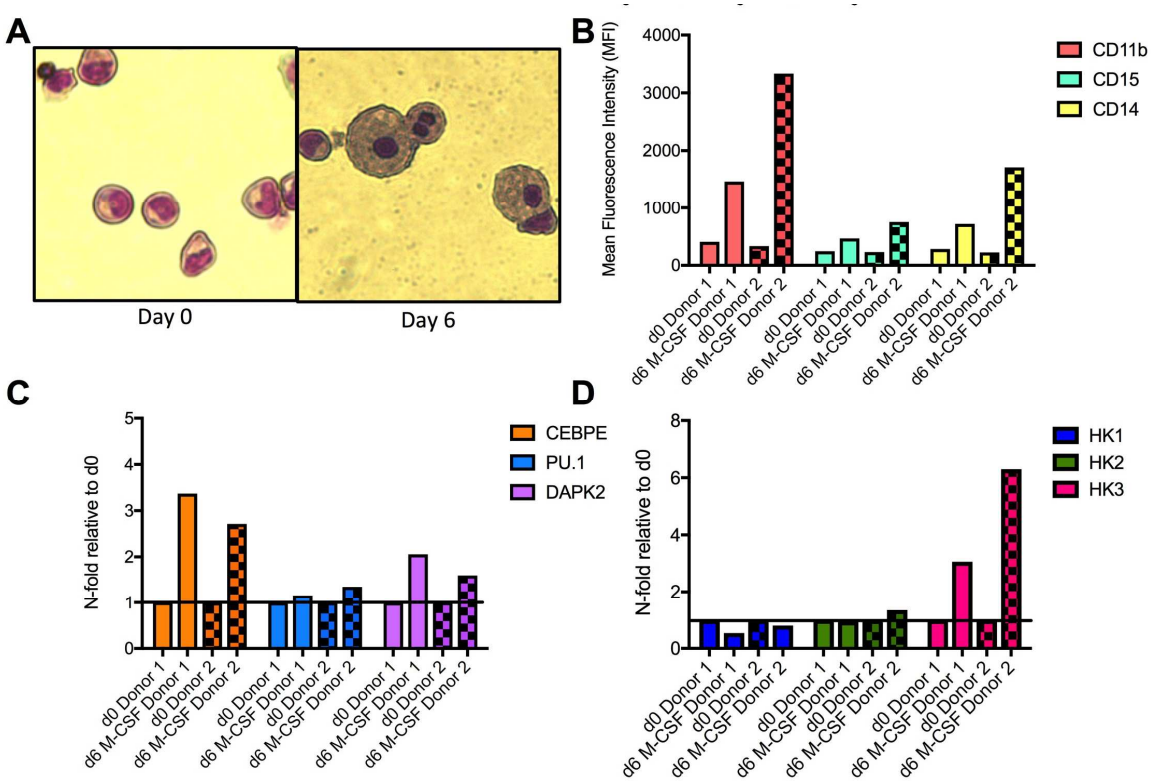

Figure 2. Increase of HK3, but not HK1 and HK2, mRNA levels after M-CSF-mediated monocyte/macrophage differentiation of CB-derived myeloid progenitor cells.
(A) Assessment of HSPC monocyte/macrophage differentiation by M-CSF treatment. $CD34^+$ HSPCs were obtained and expanded from CB of two donors. The myeloid progenitors were differentiated toward monocyte/macrophage-like cells by treatment with M-CSF, and morphological changes were assessed after day 6 by Wright-Giemsa staining. (B) Flow cytometric analysis pre- and post-M-CSF treatment. Expression levels of CD11b, CD15, and CD14 were assessed by flow cytometry of myeloid progenitor cells treated with M-CSF. (C) Quantification of mRNA expression from selected myeloid genes pre- and post-M-CSF treatment. mRNA expression of differentiation markers. mRNA expression of CEBPE, PU.1, and DAPK2 after M-CSF differentiation of $CD34^+$ cells was determined by RT-PCR. Values are normalized to the housekeeping gene GAPDH, and shown by the N-fold change compared to undifferentiated myeloid progenitor cells. (D) Quantification of HK1, HK2, and HK3 mRNA expression pre- and post-M-CSF treatment. mRNA expression of HK1, HK2, and HK3 before and after M-CSF differentiation of $CD34^+$ cells was determined by RT-PCR, and is presented as the N-fold change compared to the myeloid progenitors before M-CSF treatment. In B, C, and D the data is presented as the mean of the technical replicates of each donor; the technical replicates are within 5% of each other. (B, C, D: n=2, 2 technical replicates)

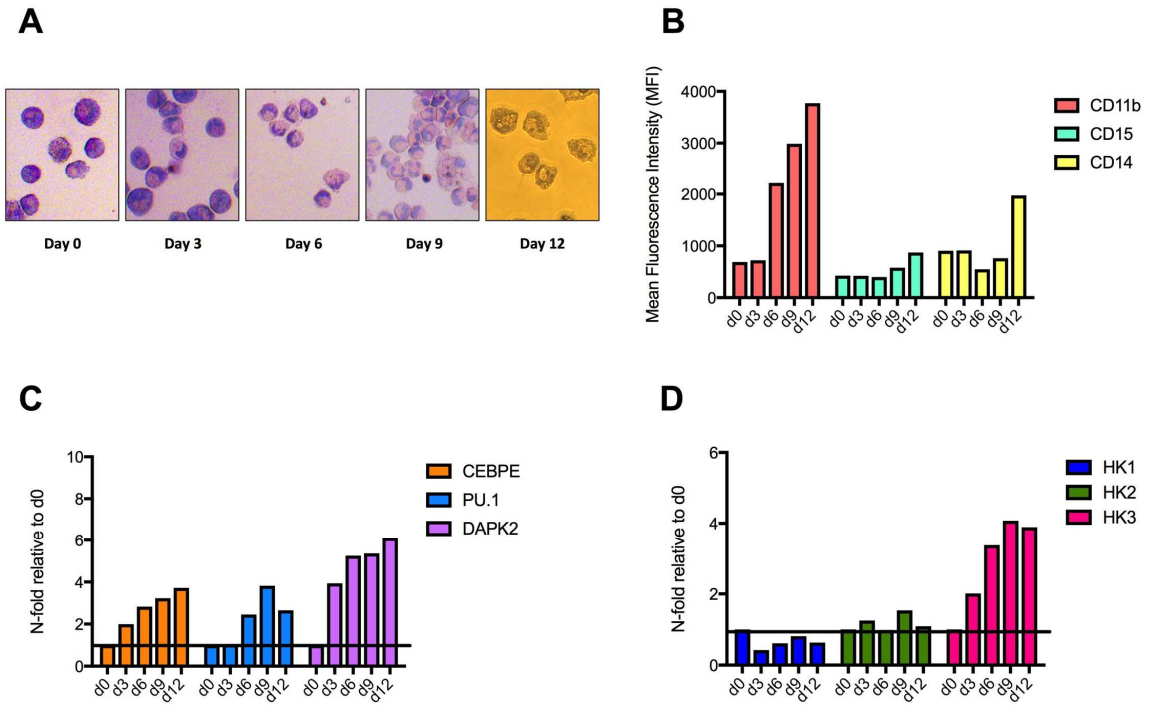

Figure 3. Increase of HK3, but not HK1 and HK2, mRNA levels during G-CSF-mediated neutrophil differentiation of MPB-derived myeloid progenitor cells.

(A) Assessment of $CD34^+$ cell differentiation by G-CSF treatment. $CD34^+$ HSPCs were obtained from MPB from one donor, and expanded for 9 and 10 days. The myeloid progenitor cells were treated with G-CSF to promote neutrophil differentiation. Morphological changes over the duration of differentiation were assessed by Wright-Giemsa staining. (B) Flow cytometric analysis of differentiation. Cell surface expression levels of CD11b, CD15, and CD14 during the course of G-CSF differentiation of myeloid progenitor cells were assessed. (C) Quantification of mRNA expression from selected myeloid genes during neutrophil differentiation. mRNA expression of CEBPE, PU.1, and DAPK2 was quantified by real-time RT-PCR. Values are normalized to the housekeeping gene GAPDH, and shown by the N-fold change as compared to myeloid progenitor cells pre-G-CSF treatment. (D) Quantification of HK1, HK2, and HK3 mRNA expression during neutrophil differentiation. mRNA expression of HK1, HK2, and HK3 were determined by RT-PCR, and presented as the N-fold change compared to the myeloid progenitors before G-CSF treatment. In B, C, D the data is presented as the mean of the technical replicates from the donor; the technical replicates are within 5% of each other. (B, C, D: n=1, 4 technical replicates)

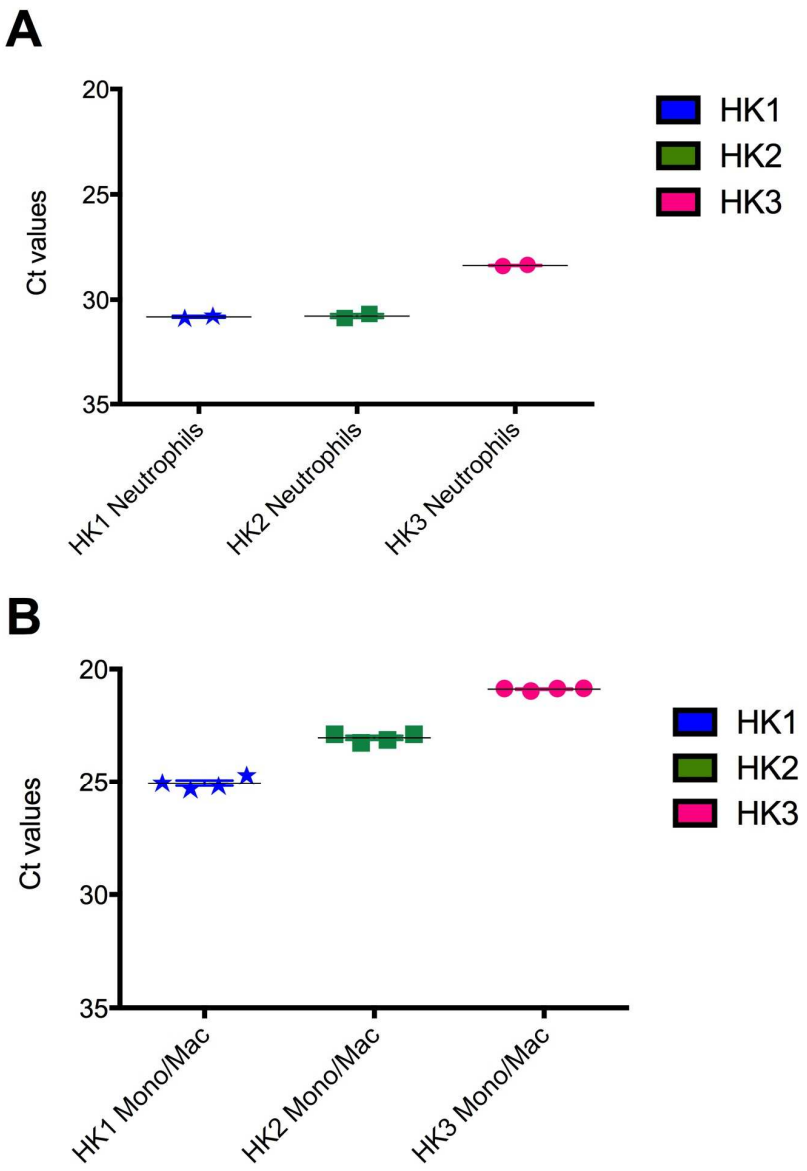

Figure 4. HK1, HK2, and HK3 expression in neutrophils and monocytes/macrophages isolated from peripheral blood.

(A) Hexokinase assessment in mature neutrophils. Neutrophils were isolated from peripheral blood from one donor and assessed for HK1, HK2, and HK3 gene expression by RT-PCR. Values are expressed in Ct values. (B) Hexokinase expression in monocytes/macrophages. Monocytes were isolated from peripheral blood from two donors, and differentiated towards macrophages. The monocyte/macrophages were assessed for HK1, HK2, and HK3 gene expression by RT-PCR. Values are expressed in Ct values. (A: n=1, 2 technical replicates; B: n=2, 2 technical replicates)

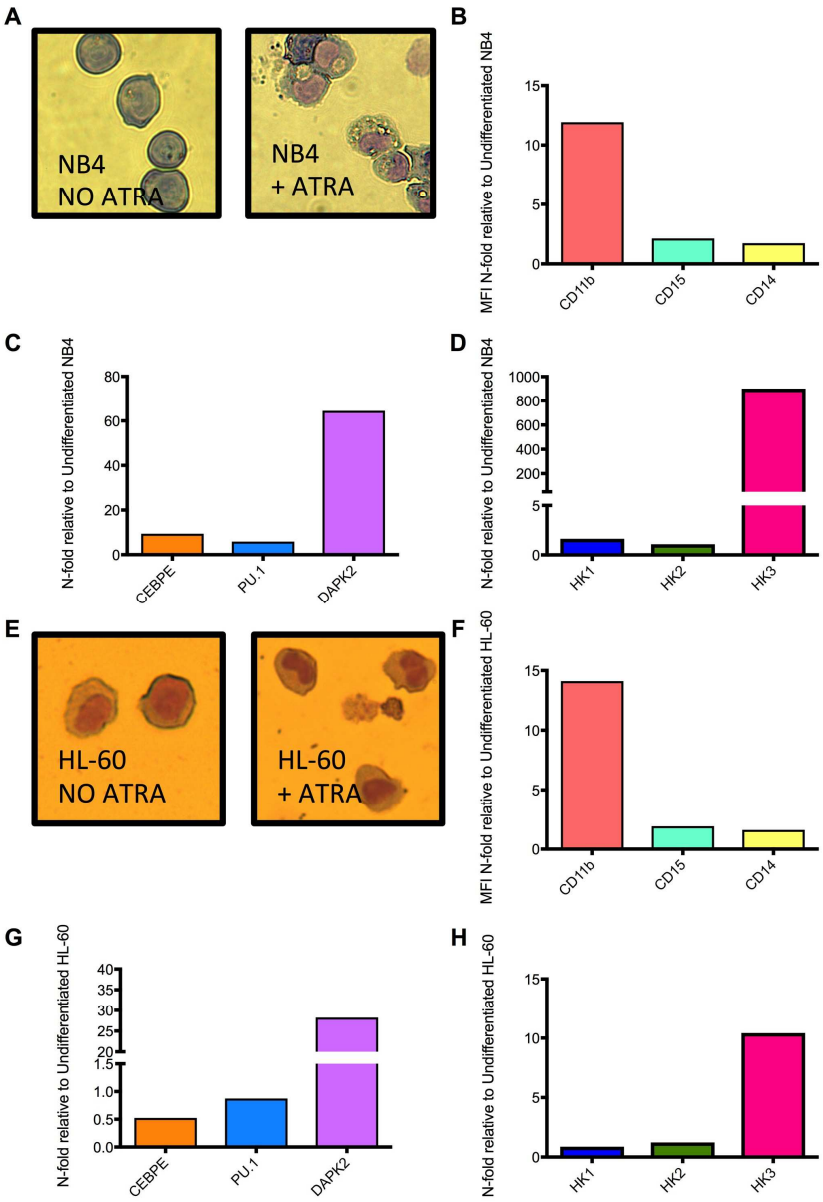

Figure 5. The NB4 and HL-60 cell lines differentiated into neutrophil-like cells by ATRA treatment have elevated expression of HK3, but not HK1 and HK2.

Morphologic assessment before and after 5μM ATRA treatment for 6-days by Wright-Giemsa staining of: (A) NB4 cells and (E) HL-60 cells. (B) Evaluation of NB4 cells and (F) HL-60 cells by flow cytometric analysis for neutrophil-like cell differentiation before and after ATRA-treatment. Expression levels of CD11b, CD15, and CD14 were assessed by flow cytometry, and are presented as the N-fold change compared to undifferentiated cells before ATRA treatment. (C) Quantification of the myeloid genes CEBPE, PU.1, and DAPK2 mRNA transcripts from NB4 cells and (G) HL-60 cells before and after ATRA-mediated differentiation. Values are normalized to the housekeeping gene GAPDH for NB4 cells and RNasep for HL-60 cells, and are presented as the N-fold change compared to undifferentiated cells. (D) Quantification of HK1, HK2, and HK3 mRNA transcripts from NB4 cells and (H) HL-60 cells before and after ATRA-mediated differentiation. HK1, HK2, and HK3 transcript levels were determined before and after ATRA treatment. The data is presented as the mean of replicates; the technical replicates are within 5% of each other. (NB4: n=2; 3 technical replicates in B, 2 technical replicates in C, D. HL-60: n=1; 3 technical replicates in F, 2 technical replicates in G, H.)

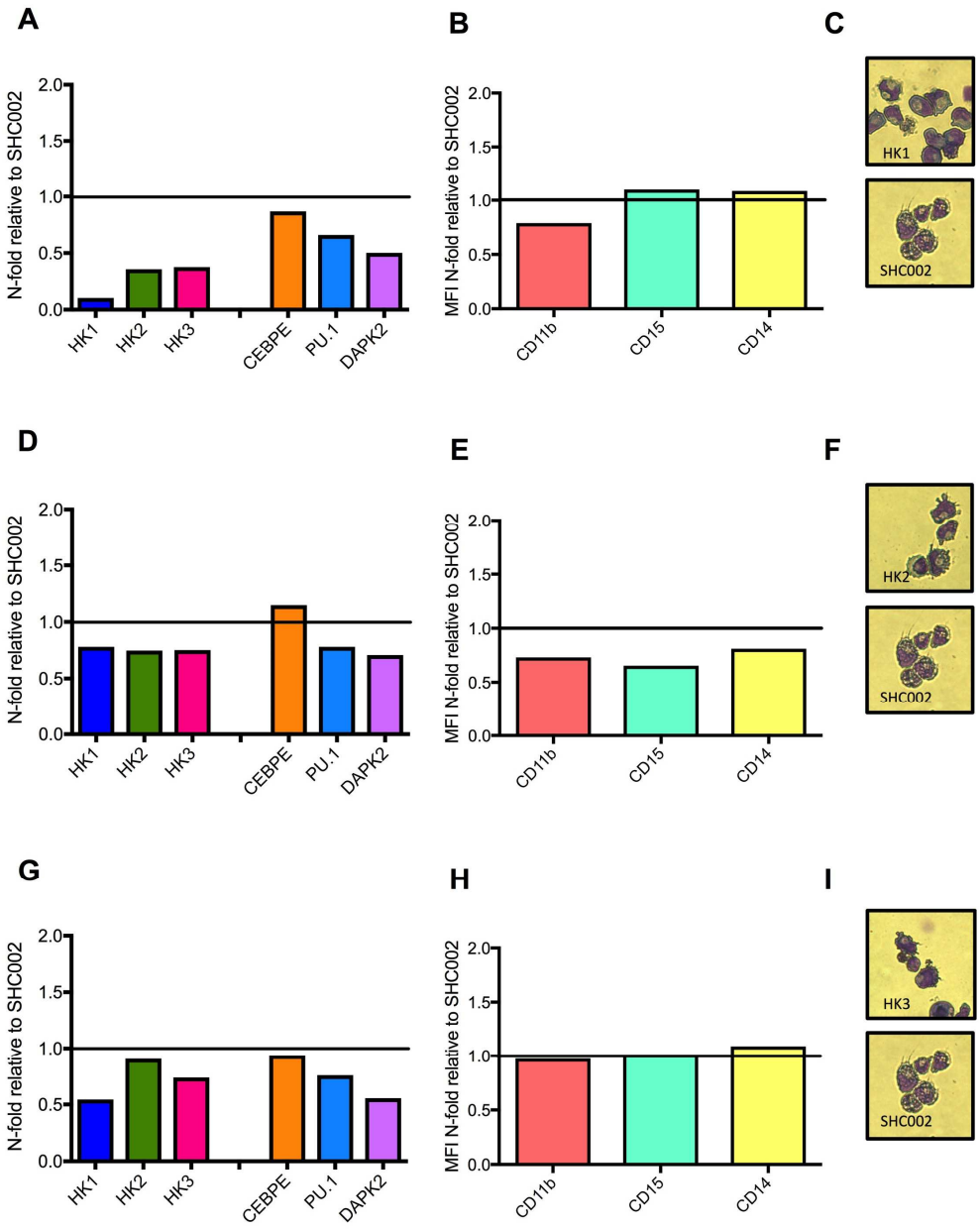

Figure 6. HK1, HK2, and HK3 knockdown NB4 cell lines.
NB4 cell lines were stably transduced with shRNAs targeting one of the three hexokinase isoforms or with a nontargeting shRNA (SHC002) as a control. HK1, HK2, and HK3 and the CEPBE, PU.1, and DAPK2 myeloid transcripts were quantified in each of the HK knockdown NB4 cell lines after differentiation with 5μM ATRA for 6-days: (A) HK1 knockdown NB4 cell line, (D) HK2 knockdown NB4 cell line, and (G) HK3 knockdown NB4 cell line. Values are normalized to the housekeeping gene GAPDH, and shown by the N-fold change compared to SHC002 control. Assessment of neutrophil differentiation of knockdown lines by flow cytometry analysis of CD11b, CD15, and CD14 cell surface expression: (B) HK1 knockdown NB4 cell line, (E) HK2 knockdown NB4 cell line, and (H) HK3 knockdown NB4 cell line were evaluated and are presented as the N-fold change compared to the SHC002 control. Morphologic changes before and after ATRA treatment were assessed by Wright-Giemsa staining: (C) HK1 knockdown NB4 cell line, (F) HK2 knockdown NB4 cell line, and (I) HK3 knockdown NB4 cell line. The data is presented as the mean of replicates; the technical replicates are within 5% of each other. (n=2, 3 technical replicates for B, E, H, 2 technical replicates for A, D, G.)

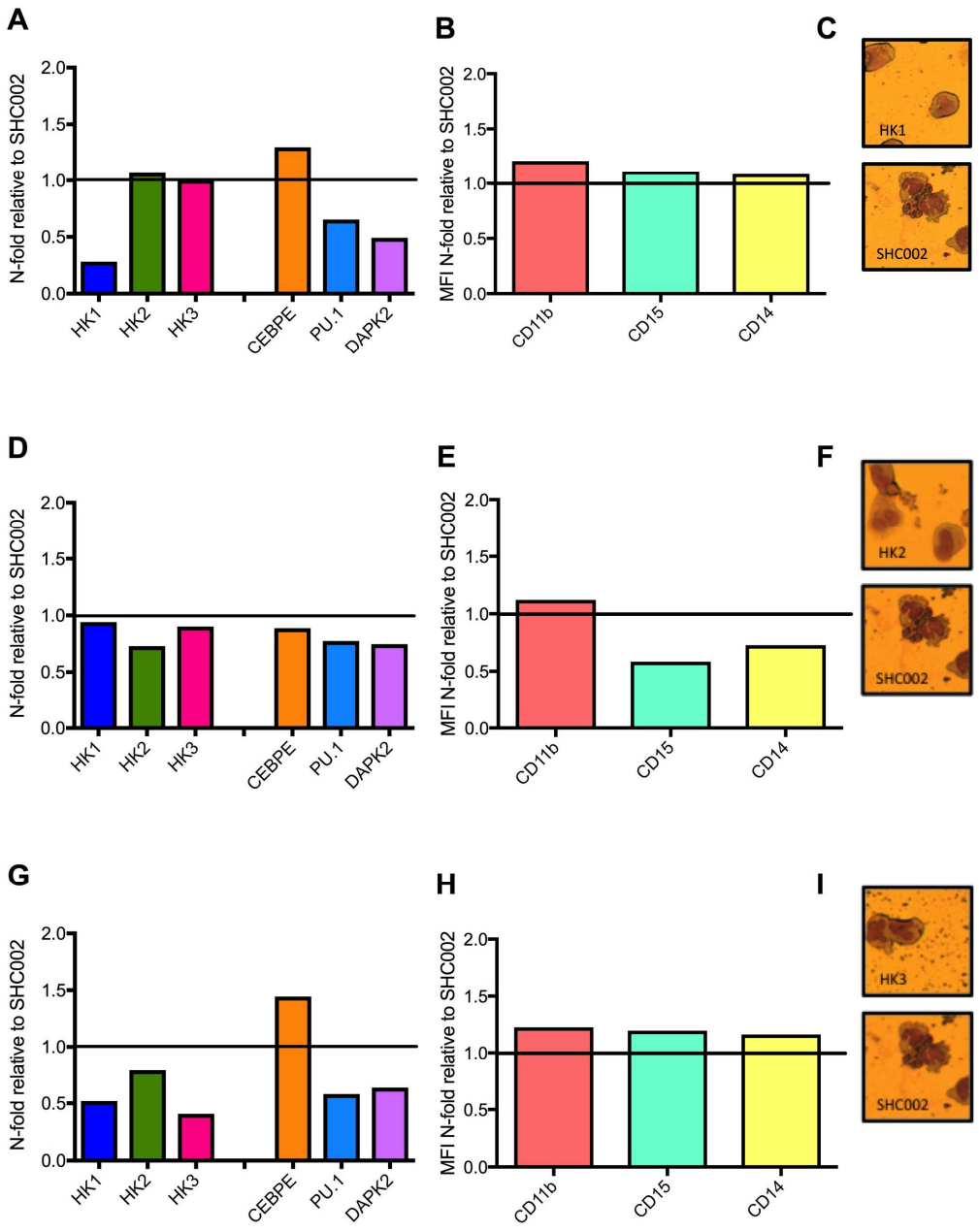

Figure 7. HK1, HK2, and HK3 knockdown HL-60 cell lines.
HL-60 cells were stably transduced with shRNAs targeting one of the three hexokinase isoforms or with a nontargeting scrambled shRNA (SHC002) as a control. HK1, HK2, and HK3 and the CEPBE, PU.1, and DAPK2 myeloid transcripts were quantified in each of the HK knockdown HL-60 cell lines after differentiation with 5μM ATRA for 6-days: (A) HK1 knockdown cell line, (D) HK2 knockdown cell line, and (G) HK3 knockdown line. Values are normalized to the housekeeping gene GAPDH, and shown by the N-fold change compared to SHC002. Assessment by flow cytometry of cell surface expression levels of CD11b, CD15, and CD14 after ATRA treatment: (B) HK1 knockdown cells, (E) HK2 knockdown cells, and (H) HK3 knockdown cells were evaluated and are presented as the N-fold change compared to the SHC002 control. Morphologic changes as the result of ATRA treatment were assessed by Wright-Giemsa staining of: (C) HK1 knockdown cells, (F) HK2 knockdown cells, and (I) HK3 knockdown cells. The data is presented as the mean of technical replicates. (n=1, 3 technical replicates for B, E, H, 2 technical replicates for A, D, G.)

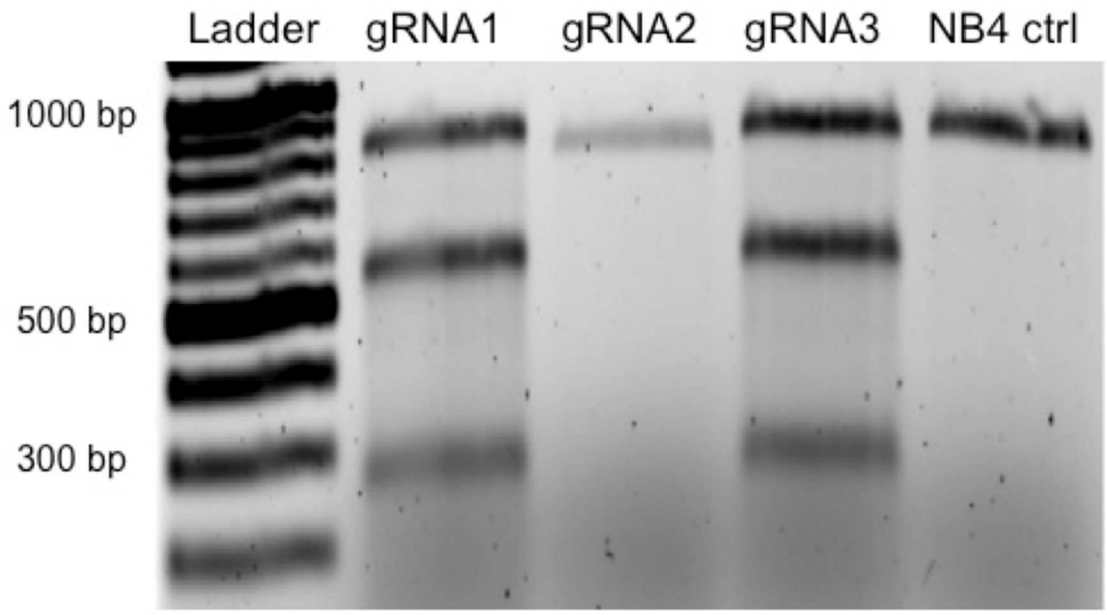

Figure 8. Assessment of HK3 gene disruption in NB4 cells utilizing the T7E1 assay.
T7E1 assay results assessing the Cas9/gRNA system for three different gRNAs targeting HK3.

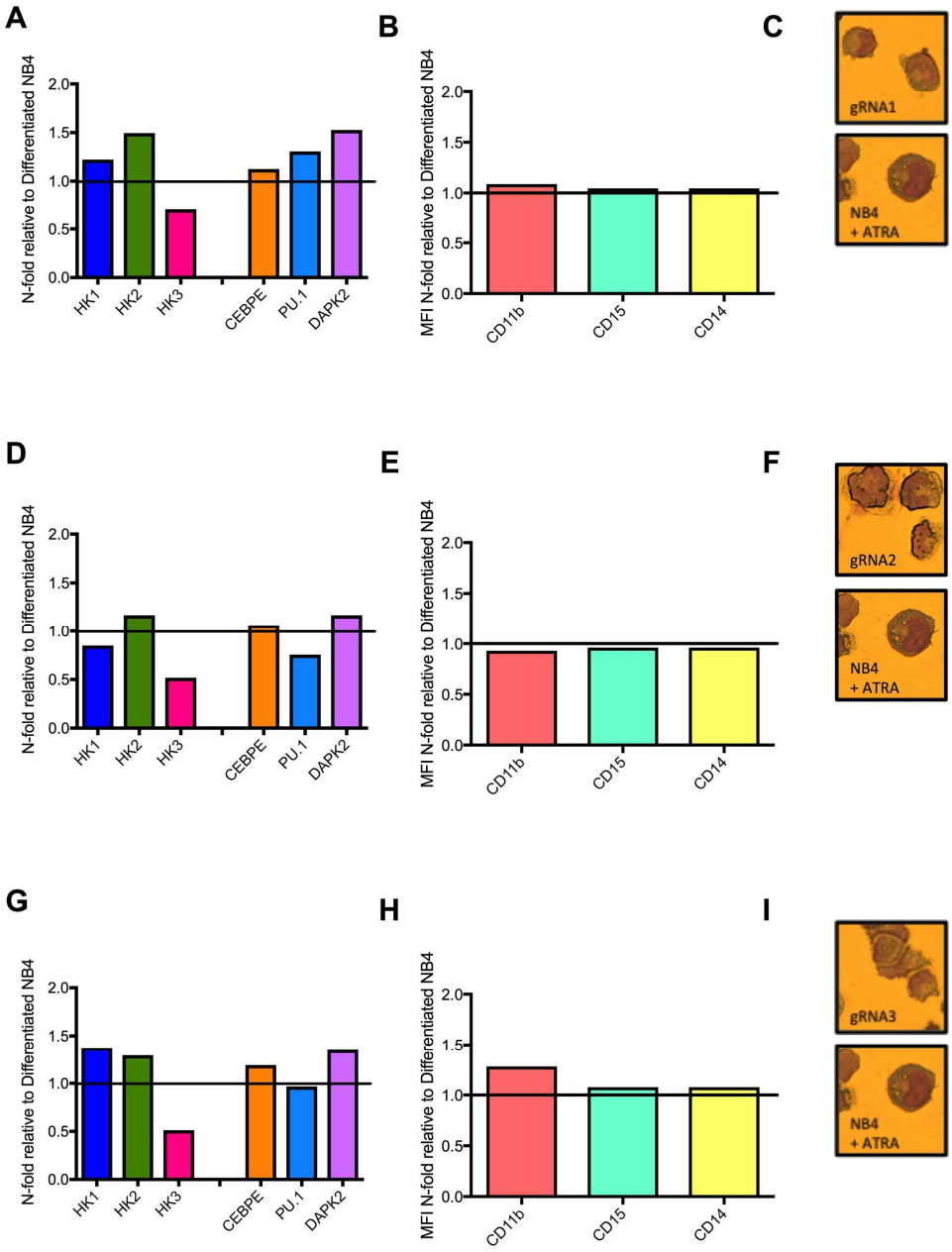

Figure 9. Assessment of NB4 cells lines expressing Cas9/gRNAs targeting HK3.
NB4 cells were stably transduced with Cas9/gRNA targeting HK3. Three independent Cas9/gRNA HK3 lines were generated, each with a unique gRNA (gRNA 1-3). Quantification of mRNA expression of HK1, HK2, and HK3 as well as the differentiation markers CEBPE, PU.1 and DAPK2 was determined by RT-qPCR after 5μM ATRA-treatment for 6-days: (A) gRNA1 HK3 cell line, (D) gRNA2 HK3 cell line, and (G) gRNA3 HK3 cell line. Values are normalized to the housekeeping gene GAPDH, and data are presented as the N-fold change compared to the ATRA treated NB4 cell line without gene editing. Expression levels of CD11b, CD15, and CD14 after ATRA-treatment of: (B) gRNA1 HK3 cell line, (E) gRNA2 HK3 cell line, and (H) gRNA3 HK3 cell line were evaluated by flow cytometry, are presented as the N-fold change compared to ATRA treated NB4 cell line without gene editing. Morphologic changes before and after ATRA-treatment were assessed by Wright-Geimsa staining of: (C) gRNA1 HK3 cell line, (F) gRNA2 HK3 cell line, and (I) gRNA3 HK3 cell line. The data is presented as the mean of technical replicates. (n=1, 3 technical replicates for B, E, H, 2 technical replicates for A, D, G.)

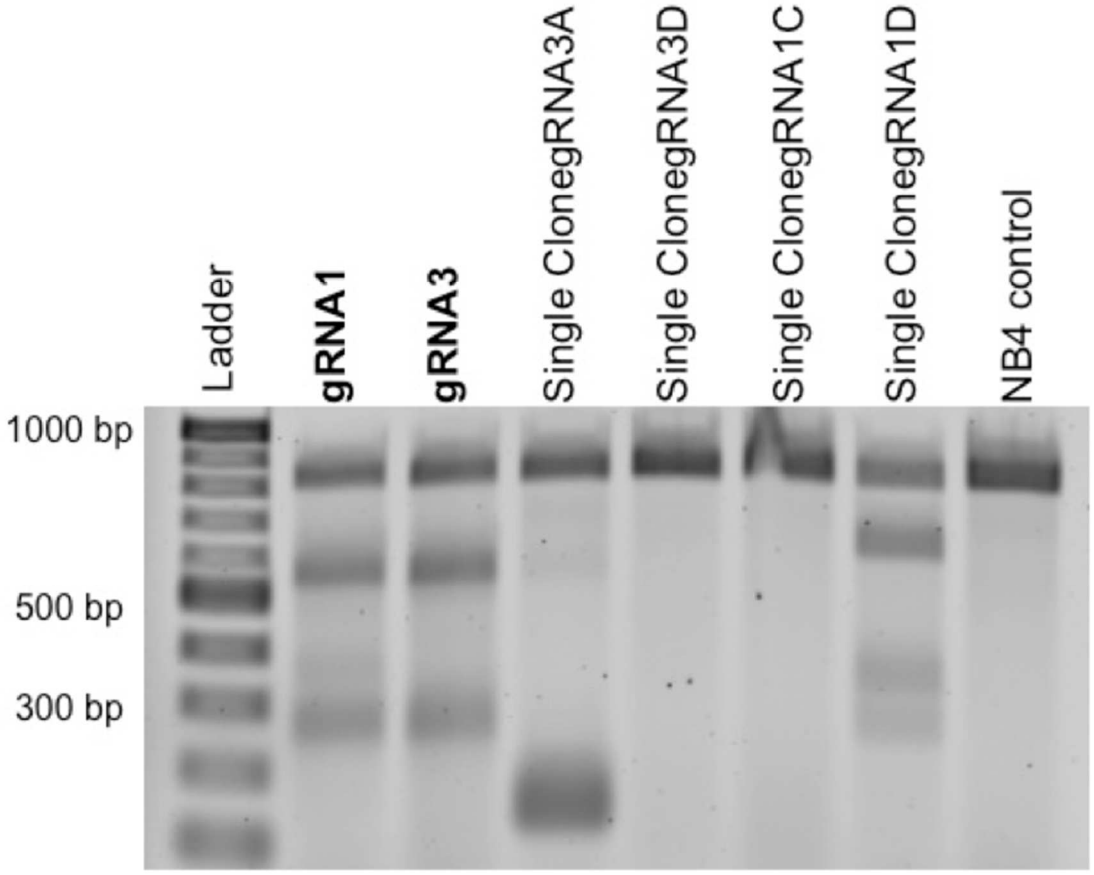

Figure 10. T7E1 assay of expressing Cas9/gRNAs targeting HK3 single clones isolated from cell lines. T7E1 assay of four single clones that were selected by limiting dilution, and analyzed by the T7E1 assay to assess mono-allelic or bi-allelic HK3 gene disruption.

DISCUSSION

In this study, I have documented increased HK3 transcript expression, but minimal changes in HK1 and HK2 transcript expression, during $CD34^+$ HSPC differentiation to neutrophils and monocytes/macrophages. HK1 and HK2 have been reported to have macrophage, dendritic cell, and neutrophil immunomodulatory functions[19], whereas HK3 has been reported to have a role in neutrophil function[15]. However, little is know about HK1-3 gene expression and their precise function in hematopoietic lineage differentiation and development. The significant increase in HK3 in neutrophil and monocyte/macrophage differentiation, as compared to HK1-2, is one step in implicating a possible role for HK3 in myeloid differentiation to neutrophils and monocytes/macrophages.

Expression of HK1-3 mRNA expression during HSPC differentiation to neutrophils and macrophages.

By investigating HK1-3 gene expression in $CD34^+$ HSPCs, I found that HK3 mRNA expression significantly increases during the duration of neutrophil differentiation in MPB HSPCs, and in neutrophils developed from neonatal CB HSPCs. In contrast, HK1 and HK2 mRNA expression remained relatively constant throughout MPB HSPC differentiation to neutrophils and in CB HSPC-derived neutrophils. The hexokinase gene expression patterns during development are independent of the developmental age of HSPCs, given that both adult and neonatal HSPCs demonstrated similar trends. It should be noted that HK mRNA expression of CB HSPC-derived neutrophils and

monocyte/macrophages was only investigated at day 6 of differentiation, due to the loss of cells after day 9, and due to low RNA quality and yield from the cells. Moreover, although mRNA patterns were indicative of distinct patterns of HK1-2 vs HK3 gene expression during differentiation, the mRNA patterns may not equate to protein patterns and therefore possibly HK function. A follow-up study to confirm whether HK protein patterns during neutrophil and monocyte/macrophage development parallels mRNA expression is warranted.

A possible explanation for the increase in HK3 mRNA expression during differentiation of neutrophils and at end-stage neutrophils may be that HK3 protein does not play a role in myeloid differentiation per se, but may be required for neutrophil function and/or survival. Neutrophils have very low rates of mitochondrial respiration and rely on glycolysis for their main source of energy in order to carry out their effector functions. Neutrophils thus contain very few mitochondria, which serves mainly for apoptotic signaling through the mitochondrial membrane potential, rather than for oxidative phosphorylation[20]. The lack of dependence on oxygen allows neutrophils to carry out immune cell functions at sites with low availability of oxygen. Previous studies of differentiation of HL-60s into neutrophil-like cells resulted in a change in metabolic preference for an increase in aerobic glycolysis and the decrease in oxidative phosphorylation due to the loss of mitochondrial supercomplex organization[21]. Compared to HK1 and HK2, which are mainly localized in the mitochondria, HK3 is cytoplasmic. Disruption of HK3 mRNA, and thus HK3 function, should help to address the question of the role of HK3 in myelopoiesis and neutrophil function and survival.

The studies on HK gene expression of CB HSPCs developed to monocytes/macrophages showed a similar pattern of HK transcript expression as was seen for neutrophils. Thus, low levels of HK1 and HK2 transcript expression and increased HK3 transcript expression appears to be a consequence of myelopoiesis cumulating in end-stage neutrophils and monocytes/macrophages. Whether HK3 is required for monocytes/macrophages differentiation and/or function remains to be determined.

Preliminary studies on HK transcript expression in developed neutrophils and monocytes/macrophages isolated from peripheral blood showed similar patterns of HK transcript expression as was found in neutrophils and monocytes/macrophages *in vitro*, low levels of HK1 and HK2 transcripts relative to HK3. The mRNA expression of hexokinase was quantified using RT-qPCR. Due to the lack of an appropriate housekeeping gene and the small yield of RNA from neutrophils and macrophages, the calculation of the relative N-fold change by the equation $2^{-\Delta\Delta Ct}$ could not be used. Therefore, Ct values were used to compare hexokinase gene expression for neutrophils and monocytes/macrophages. However, variables associated with RT-qPCR cannot be normalized without the housekeeping gene, such as variations arising from sample preparation and handling, RNA isolation, reverse transcription efficiency, and PCR reaction amplification efficiencies. The housekeeping gene HMBS (hydroxymethylbilane synthase) used was not suitable since it was not stably expressed before and after differentiation. Due to stable expression of GAPDH throughout differentiation of HSPCs, GAPDH was used as the housekeeping gene for the other primary cell studies to obtain normalized gene expression. GAPDH is not the optimal housekeeping gene to use in

studies associated with metabolism, as GAPDH is a glycolytic enzyme that catalyzes the sixth step of glycolysis. Thus, a follow up study to confirm the HK gene expression patterns on the protein level is warranted.

The increase of HK3 transcripts during neutrophil and monocyte/macrophage differentiation and in end-stage myeloid cells might suggest that there is a cellular mechanism for increased glycolysis required for differentiation and/or terminal myeloid cell function. Given that HK3 appears to be localized to end-stage myeloid cells, as compared to the ubiquitous cellular expression of HK1 and HK2[11], and that HK3 is regulated by the PU.1 myeloid transcription factor[15], the presence of HK3 may reflect a functional role in neutrophils and monocytes/macrophages. Similar to how the inhibition of glycolysis directs erythropoiesis, the promotion of glycolysis through increased levels of HK3 may contribute to myelomonocytic differentiation. Interestingly, PU.1 is decreased during erythroid differentiations compared to neutrophil and macrophage differentiation where PU.1 is upregulated[7,22,23,24].

HK expression in the promyelocyte cell lines NB4 and HL-60 during ATRA-mediated differentiation and generation of HK knockdown cell lines.

The NB4 and HL-60 cell lines differentiated to neutrophil-like cells by ATRA demonstrated similar HK transcript expression patterns as were observed in MBP and CB HSPCs differentiated to neutrophils by G-CSF. The similar pattern of HK transcript expression in NB4 and HL-60 cells, as seen in primary neutrophil differentiation, and the ease of use of the cell lines provides an ideal cell model for investigating the effect of HK gene disruption on neutrophil differentiation. Moreover, my findings on HK3 transcript

expression in ATRA-differentiated NB4 cells of HK mRNA transcript patterns were similar to an earlier report by Federzoni, et al.[15], although the relative fold HK3 transcript levels were higher than what was determined from our NB4 cell line studies.

The lentiviral delivery of shRNAs targeting each of the hexokinase mRNAs in NB4 and HL-60 cell lines resulted in stable shRNA expression and individual cell lines with differences in HK mRNA knockdown efficacy and inconclusive results of the effects upon ATRA-mediated differentiation. The disruption of any of the HK isoforms in both NB4 and HL-60 cell lines resulted in decreased gene expression of PU.1, the master regulator of myeloid differentiation and function, and DAPK2, which suggests hindrance in neutrophil differentiation upon ATRA-treatment. However, the decrease in PU.1 and DAPK2 transcripts conflicted with the changes observed in CEBPE transcript. CEBPE gene expression in PU.1 knockdowns has been shown to be decreased relative to the wild type and has been used previously in assessing granulocyte differentiation. However, in my findings, CEBPE gene expression did not correlate with the observed decrease in PU.1 gene expression. CEBPE expression was unchanged in NB4 shRNA cell lines; CEBPE was slightly increased in HL-60 shRNA cell lines that had altered expression of HK 1, 2, 3 and PU.1. Changes in surface marker expression of CD11b did not correlate with the observed decreases in expression of PU.1 and DAPK2. CD11b, which is regulated by PU.1, is a cell surface marker commonly used to assess ATRA-induced neutrophil differentiation studies in NB4 and HL-60 cells. Changes in CD11b expression were insignificant across both NB4 and HL-60 shRNA cell lines, indicating that neutrophil differentiation was not affected, despite the observed decreases in PU.1. In addition, the other surface marker CD15, a myeloid gene that is not regulated by the

PU.1, also had no observed significant changes in the cell lines with disruptions in HK transcript. In conclusion, the surface and gene markers for myeloid differentiation had conflicting results on the extent of neutrophil differentiation of the HK shRNA cell lines. The efficacy of the disruption of HK transcript may have been insufficient to observe effects on neutrophil differentiation. To further investigate the role of HK in neutrophil differentiation, a different approach focusing on the HK3 isozyme was undertaken.

Generation of HK3 knockout cell lines utilizing CRISPR/Cas9.

All three generated HK3 gene-edited NB4 cell lines had a 30-50% decrease in HK3 transcripts. The altered expression of HK3 had minor effects on HK1 and HK2 gene expression across all three cell lines. Based on a previous report on HK3 knockdown NB4 cell line which demonstrated disruption of neutrophil differentiation with ATRA-treatment, it might be expected that upon gene editing of HK3 a similar phenotype would result[15]. However, gene-edited NB4 cell lines with 30-50% disrupted HK3 gene expression resulted in minor changes in neutrophil differentiation; there were no observable changes in gene expression of PU.1, DAPK2, and CEBPE, as well as the surface expression of CD11b and CD15. This result is likely due to a heterogeneous population consisting of cells containing HK3 alleles with complete functional knockouts, alleles with partial loss of function, and wild-type alleles.

In order to obtain a homogeneous HK3 knockout cell line for use, individual single clones will have to be selected and assessed for disruption of HK3 expression. The extent of the HK3 knockout depends on whether the CRISPR/Cas9-induced mutation results in a loss-of-function of the protein. Unless the mutation results in a premature stop

codon, RT-PCR will detect HK3 mRNA. Therefore, protein expression analysis and functional assays are required for the validation of the HK3 CRISPR gRNA cell lines.

Future Directions.

The identification of increased HK3 expression in myelopoiesis of CB and MPB HSPCs implicates the role of HK3 in neutrophil and monocyte/macrophage differentiation or function. Once the homogeneous HK3 knockout cell line is generated, ATRA differentiation studies would reveal if the isozyme is involved in neutrophil differentiation. Seeing that the surface marker and mRNA gene expression of myeloid genes used in assessing neutrophil differentiation might not be a suitable fine-grained analysis of neutrophil differentiation, a profile of global changes in protein expression based on mass spectrometry may provide better insights into the effects of knocking out HK3.

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SUPPLEMENTARY MATERIAL

Table S1- CRISPR Guide RNA Sequences for HK3 and HK2

gRNA	Sequence
HK3_gRNA1	CTGGAGCTGGGGGCCAC
HK3_gRNA2	GGATGCTGCCTACATACGTG
HK3_gRNA3	GTCACCAGACGGGCTTGGAC
HK2_gRNA1	TGACCACATTGCCGAATGCC
HK2_gRNA2	TAGCCAGGCATTCGGCAATG
HK2_gRNA3	TTACCTCGTCTAGTTTAGTC

Table S2- PCR Primers

Primer Set	Sequence
HK3_gRNA1	*F:* CACCGCTGGAGCTGGGGGCCAC *R:* AAACGTGGCCCCCAGCTCCAGC
HK3_gRNA2	*F:* CACCG GGATGCTGCCTACATACGTG *R:* AAAC CACGTATGTAGGCAGCATCC C
HK3_gRNA3	*F:* CACCG GTCACCAGACGGGCTTGGAC *R:* AAAC GTCCAAGCCCGTCTGGTGAC C
HK2_gRNA1	*F:* CACCGTGACCACATTGCCGAATGCC *R:* AAACGGCATTCGGCAATGTGGTCAC
HK2_gRNA2	*F:* CACCGTAGCCAGGCATTCGGCAATG *R:* AAACCATTGCCGAATGCCTGGCTAC
HK2_gRNA3	*F:* CACCGTTACCTCGTCTAGTTTAGTC *R:* AAACGACTAAACTAGACGAGGTAAC
T7E1 HK3_gRNA1,3	*F:* ATGGCACTGGTGGGTATCAT *R:* CTCTCTCAGAAGCCCAGACC
T7E1 HK3_gRNA2	*F:* GGTGTATGAAGAGCCGAGGA *R:* AGCTGCAGACCCTGTTTGTT