PIEZO1 GAIN-OF-FUNCTION VARIANTS LEAD TO ALTERATIONS IN LATE-STAGE ERYTHROPOIESIS BY ENHANCING ENUCLEATION RATE

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INTRODUCTION

PIEZO1 is a mechanosensitive cation channel involved in various physiological processes as a sensor of mechanical forces. In erythrocytes, it is involved in the regulation of hydration and cellular volume. Gain-of-function (GoF) mutations in PIEZO1 slow the inactivation kinetics of the channel leading to dehydrated hereditary stomatocytosis (DHS, or xerocytosis).(1) Despite the variable phenotypes, most patients present mild to severe hemolytic anemia, and iron overload.(2 Erythroid progenitor cells of DHS patients showed alterations of erythroid differentiation. (3-4) Both the constitutive and macrophage GoF Piezo1 mice demonstrated that PIEZO1 is a key regulator of macrophage phagocytic activity and subsequent erythrocyte turnover. (5)

AIM

In this study, we investigated the specific role of PIEZO1 in altered erythropoiesis in DHS by an *in vitro* model of erythroid precursors during to erythroid differentiation.

RESULTS

We generated an erythroid model of DHS using Human Umbilical cord blood-Derived Erythroid Progenitor-2 (HUDEP-2) cells, by inserting the PIEZO1 GoF variant, R2456H, in the heterozygous state by CRISPR/Cas9. The resulting cell line, HUDEP2-PIEZO1-KI (PIEZO1-KI), was induced to erythroid differentiation for 10 days by erythropoietin and compared to HUDEP2-WT cells. We tested for erythroid surface marker expression by flow cytometry. Combined and individual analyses of CD36 and Glycophorin A (GPA) showed that PIEZO1-KI cells presented an increase in late erythroblasts. By day 7, over 50% of PIEZO1-KI cells differentiated into late erythroblasts, whereas PIEZO1-WT cells still presented intermediate erythroblasts **(Figure 1A,B)**. Single-marker analysis confirmed a significant increase in GPA positive cells (a marker of late erythroblasts) in PIEZO1-KI cells compared to WT cells and no differences in CD36-positive cells (early erythroid markers) **(Figure 1C)**. Hoechst/GPA co-staining revealed a higher percentage of GPA-positive/Hoechst-negative cells in PIEZO1-KI from day 7 compared to WT cells , confirming an accelerated differentiation that results in an increased enucleation rate. **(Figure D,E)**

Figure 1

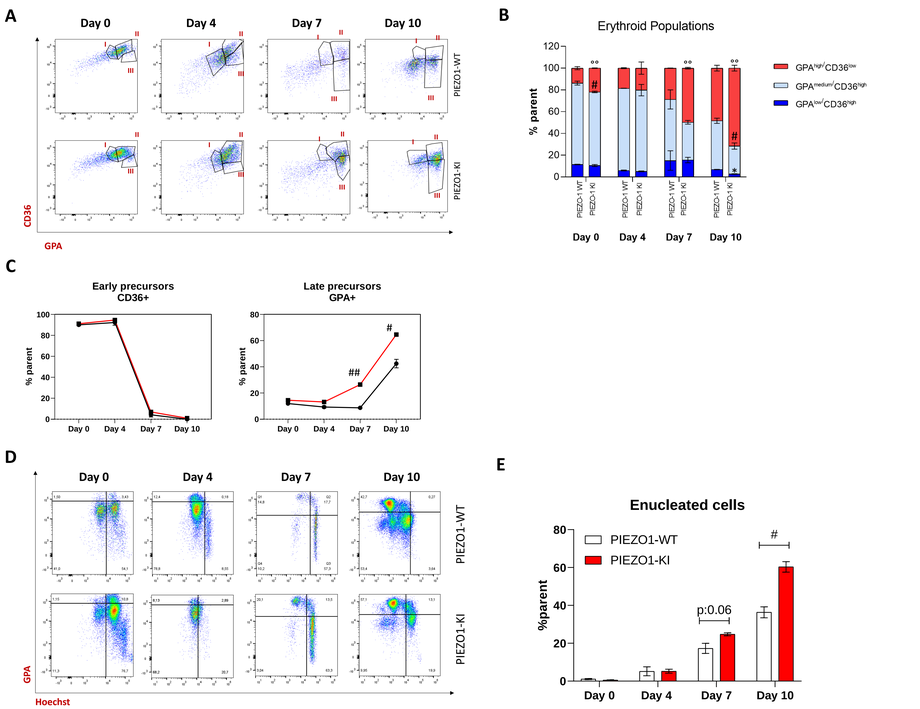


Figure 1. Flow cytometry analysis reveals accelerated terminal differentiation and increased enucleation rate. (A) Representative plots of FACS analysis showing Hudep-2 PIEZO1-WT (upper) and PIEZO1-KI (lower) at day 0, 4, 7, 10 of erythroid differentiation. Differentiating cells are separated into three populations ($Gly^{low}/CD36^{high}$, $Gly^{medium}/CD36^{medium}$, $Gly^{high}/CD36^{low}$) basing on **CD36 and GPA** levels. **(B)** Histograms showing the percentage of differentiating population of Hudep-2 PIEZO1-WT (upper) and PIEZO1-KI (lower) at day 0, 4, 7, 10 of erythroid differentiation. *p<0,05 PIEZO1-KI vs PIEZO1-WT (**$Gly^{low}/CD36^{high}$**); #p<0,05 PIEZO1-KI vs PIEZO1-WT (**$Gly^{medium}/CD36^{high}$**) °° **p** <0,01 PIEZO1-KI vs PIEZO1-WT (**$Gly^{high}/CD36^{low}$**) by Multiple-t-test. ($Gly^{low}/CD36^{high}$, $Gly^{medium}/CD36^{medium}$, $Gly^{high}/CD36^{low}$) **(C)** Dot plot showing **CD36 levels** (*left*) and **GPA** levels (*right*) at day 0, 4, 7, 10 of erythroid differentiation in Hudep-2 PIEZO1-WT (black) and PIEZO1-KI (red). # p<0,05 PIEZO1-KI vs PIEZO1-WT, ## p<0,01 PIEZO1-KI vs PIEZO1-WT by Multiple-t-test. **(D)** Representative plots of FACS analysis showing **Hoechst** and **GPA** levels of Hudep-2 PIEZO1-WT (upper) and PIEZO1-KI (lower) at day 0, 4, 7, 10 of erythroid differentiation**. E)** Histograms showing the percentage of PIEZO1-WT (withe) and PIEZO1-KI (red) Hudep-2 cells at each day of differentiation. Enucleated cells are defined basing on **high GPA** and **low Hoechst**. # p<0,05 PIEZO1-KI vs PIEZO1-WT, by Multiple-t-test.

To further characterize the erythroid differentiation, RNAseq was performed on day 0, 7, and 10 of differentiation. We firstly focused on genes physiologically regulated during differentiation of WT cells (359 genes). Among them, we selected both genes with the **same regulation trend** (**List_1: n=258**) and with a **different regulation trend** in PIEZO1-KI cells compared to WT (**List_2: n=101**). Gene ontology on genes of list_1 revealed, as expected, "erythrocyte development" as the most enriched biological process (BP). Particularly, single gene analysis revealed that three genes, although presenting the same regulation trend, are significantly downregulated in PIEZO1-KI cells at day 10 of differentiation: ALAS2 (heme formation), DMTN (erythrocyte shape regulation), and BPGM (hemoglobin oxygen affinity regulation and glycolysis). **(Figure 2A,B)** Gene ontology on genes of list_2, besides, revealed alteration of genes involved in glycolytic process. Particularly, we found three genes to have different regulation trends: PFKM (involved in the first step of glycolysis) was suppressed during differentiation, while PGAM1 and ENO3 (conversion of 3-phosphoglycerate to phosphoenolpyruvate) were upregulated. **(Figure 2C,D)**

Figure 2

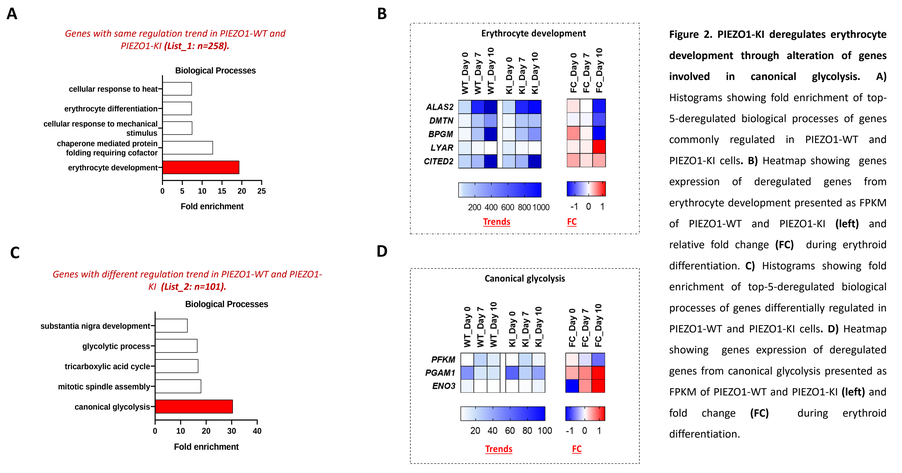
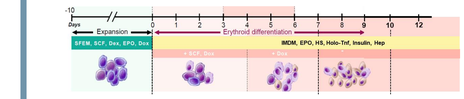


Figure 2. PIEZO1-KI deregulates erythrocyte development through alteration of genes involved in canonical glycolysis. A) Histograms showing fold enrichment of top-5-deregulated biological processes of genes commonly regulated in PIEZO1-WT and PIEZO1-KI cells**. B)** Heatmap showing genes expression of deregulated genes from erythrocyte development presented as FPKM of PIEZO1-WT and PIEZO1-KI **(left)** and relative fold change **(FC)** during erythroid differentiation. **C)** Histograms showing fold enrichment of top-5-deregulated biological processes of genes differentially regulated in PIEZO1-WT and PIEZO1-KI cells**. D)** Heatmap showing genes expression of deregulated genes from canonical glycolysis presented as FPKM of PIEZO1-WT and PIEZO1-KI **(left)** and fold change **(FC)** during erythroid differentiation.

METHODS

- In-vitro differentiation of engineered Hudep2-PIEZO-R2456H (PIEZO1-KI) in a 3-phase liquid culture protocol.



- Flow cytometry of erythroid differentiation markers
- RNA-sequencing

CONCLUSIONS

Herein we demonstrated that the PIEZO1 GoF variants induce an accelerated erythropoiesis, particularly affecting the late stages of erythroid differentiation, and altering the enucleation rate of differentiating erythroblasts. The altered erythroid differentiation processes is accompanied by alteration of genes involved in glycolytic pathway. These in vitro findings link several components of glycolysis to changes in erythroid differentiation caused by a prototypical PIEZO1 GoF mutation in DHS. These findings suggest future studies should investigate the potential benefits of modulating glycolysis in DHS models.

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