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PIEZO1 gain-of-function drives glycolytic imbalance in late-stage erythropoiesis: the potential of mitapivat therapy in dehydrated hereditary stomatocytosis

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INTRODUCTION

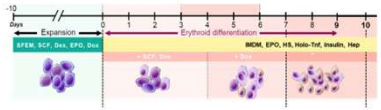
PIEZO1 is a mechanosensitive cation channel that plays a crucial role in various physiological processes as a mechanical force sensor. Gain-of-function (GoF) mutations in PIEZO1 lead to dehydrated hereditary stomatocytosis (DHS; also referred to as hereditary xerocytosis) by slowing PIEZO1 inactivation kinetics. DHS patients exhibit mild to severe hemolytic anemia, as well as hepatic iron overload. Erythroid progenitor cells from DHS patients show mutation-dependent alterations in erythroid differentiation. Both constitutive and macrophage-specific GoF Piezo1 mice showed mild anemia and demonstrated Piezo1 involvement in erythrocyte turnover.

AIM

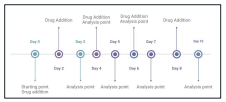
To investigate the specific role of PIEZO1 in erythroid differentiation and the potential benefits of mitapivat treatment, an allosteric activator of pyruvate kinase, a key enzyme of the glycolysis, in PIEZO1-mediated dyserythropoiesis.

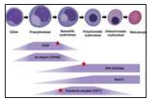
METHODS

***In vitro* differentiation** of **engineered** human umbilical cord blood-derived erythroid progenitor 2 (**Hudep-2**) **PIEZO-R2456H** (PIEZO1-Knock In (KI)) in a three-phase liquid culture protocol.



Drug treatment: Mitapivat (1uM) was added to cells each two days; **Flow cytometry** was performed at selected analysis points on glycophorin A (GPA), CD36, CD49d, CD71, and Hoechst for nucleus staining.





RNA sequencing (RNAseq) was performed on day 0, 7, and 10 of erythroid differentiation. Analysis was performed considering genes with log2fold changes ≥ ±1 and log10pValue ≥ 1,3.Pathway analysis was performed by Gene Ontology.

RESULTS

PIEZO1-WT and PIEZO1-KI cells were induced to erythroid differentiation and tested for erythroid surface marker expression by flow cytometry. Combined and individual analyses of CD36 and GPA showed that PIEZO1-KI cells presented an increase in late erythroblasts **(Late-E)**. By day 7, over 50% of PIEZO1-KI cells differentiated into Late-E, whereas PIEZO1-WT cells still presented intermediate erythroblasts **(Intermediate-E) (Figure 1A,B)**. Single-marker analysis confirmed a significant increase in GPA positive cells (a marker of late erythroblasts) in PIEZO1-KI cells no differences in CD36-positive cells (markers of early erythroblasts (**Early-E**)) **(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)**

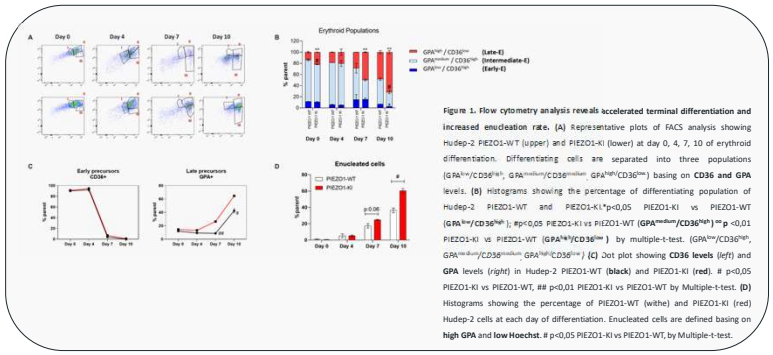


Figure 1. Flow cytometry analysis reveals accelerated terminal differentiation and increased enucleation rate. (A) Representative plots of FACS analysis showing Hudep-2 PIEZO1-WT (upper) and PIEZO1-KI (lower) at day 0, 4, 7, 10 of erythroid differentiation. Differentiating cells are separated into three populations ($GPA^{low}/CD36^{high}$, $GPA^{medium}/CD36^{medium}$, $GPA^{high}/CD36^{low}$) basing on **CD36 and GPA** levels. **(B)** Histograms showing the percentage of differentiating population of Hudep-2 PIEZO1-WT and PIEZO1-KI.*p<0,05 PIEZO1-KI vs PIEZO1-WT ($GPA^{low}/CD36^{high}$); #p<0,05 PIEZO1-KI vs PIEZO1-WT ($GPA^{medium}/CD36^{high}$) ** p <0,01 PIEZO1-KI vs PIEZO1-WT ($GPA^{high}/CD36^{low}$) by multiple-t-test. ($GPA^{low}/CD36^{high}$, $GPA^{medium}/CD36^{medium}$ $GPA^{high}/CD36^{low}$) **(C)** Dot plot showing **CD36 levels** (*left*) and **GPA** levels (*right*) 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)** 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 understand molecular mechanisms underlying alteration in erythropoiesis, a transcriptomic analysis was performed. We focused on genes physiologically regulated during differentiation of WT cells (359 genes) selecting genes with a **different regulation trend** in PIEZO1-KI cells compared to WT (**n=101**). Gene ontology analysis 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 2A,B)**

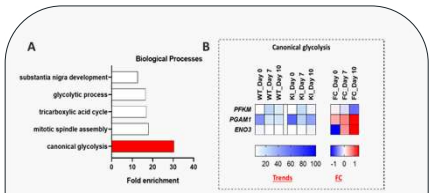


Figure 2. PIEZO1-KI deregulates erythrocyte development through alteration of genes involved in canonical glycolysis. A) Histograms showing fold enrichment of top-5-deregulated biological processes of genes differentially regulated in PIEZO1-WT and PIEZO1-KI cells. **B)** Heatmap showing genes expression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

RESULTS

To test whether modulating glycolysis could rescue erythroid differentiation defect, PIEZO1-KI cells were treated with mitapivat.. Evaluation of differentiating populations by combined FACS analysis (GPA and CD49d), revealed that mitapivat restored the differentiation pattern of PIEZO1-KI cells closer to the physiological process. **(Figure 3A,B)** Single marker analysis confirmed that mitapivat treatment reduces the percentage of GPA^+ cells to WT levels and normalizes CD71 levels along differentiation. **(Figure 3C)** Besides, we evaluated how enucleation is affected by mitapivat treatment. Surprisingly, we found that at day 10 of differentiation, mitapivat significantly decreased the differentiated populations ($Hoechst^-/GPA^+$)while increasing intermediate precursors ($Hoechst^-/GPA^-$) **(Figure 3D,E)**

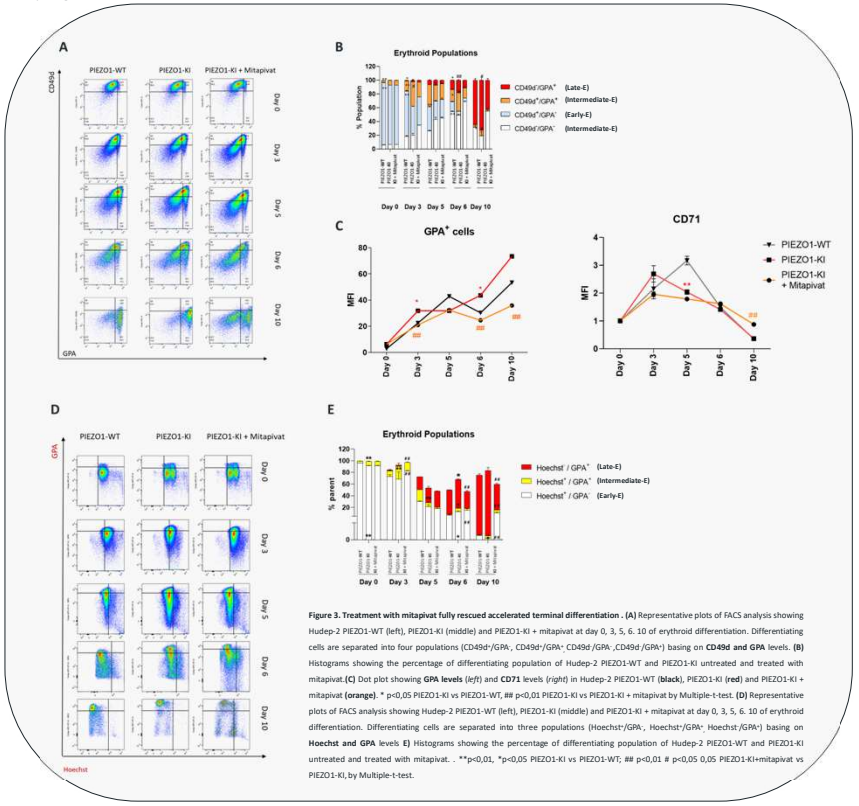


Figure 3. Treatment with mitapivat fully rescued accelerated terminal differentiation . (A) Representative plots of FACS analysis showing Hudep-2 PIEZO1-WT (left), PIEZO1-KI (middle) and PIEZO1-KI + mitapivat at day 0, 3, 5, 6. 10 of erythroid differentiation. Differentiating cells are separated into four populations ($CD49d^+/GPA^-$, $CD49d^+/GPA^+$ $CD49d^-/GPA^-$,$CD49d^-/GPA^+$) basing on **CD49d and GPA** levels. **(B)** Histograms showing the percentage of differentiating population of Hudep-2 PIEZO1-WT and PIEZO1-KI untreated and treated with mitapivat.**(C)** Dot plot showing **GPA levels** (*left*) and **CD71** levels (*right*) in Hudep-2 PIEZO1-WT (**black**), PIEZO1-KI (**red**) and PIEZO1-KI + mitapivat (**orange**). * p<0,05 PIEZO1-KI vs PIEZO1-WT, ## p<0,01 PIEZO1-KI vs PIEZO1-KI + mitapivat by Multiple-t-test. **(D)** Representative plots of FACS analysis showing Hudep-2 PIEZO1-WT (left), PIEZO1-KI (middle) and PIEZO1-KI + mitapivat at day 0, 3, 5, 6. 10 of erythroid differentiation. Differentiating cells are separated into three populations ($Hoechst^+/GPA^-$; $Hoechst^+/GPA^+$ $Hoechst^-/GPA^+$) basing on **Hoechst and GPA** levels **E)** Histograms showing the percentage of differentiating population of Hudep-2 PIEZO1-WT and PIEZO1-KI untreated and treated with mitapivat. . **p<0,01, *p<0,05 PIEZO1-KI vs PIEZO1-WT; ## p<0,01 # p<0,05 0,05 PIEZO1-KI+mitapivat vs PIEZO1-KI, by Multiple-t-test.

CONCLUSIONS

Taken together, these findings link several components of glycolysis to changes in erythroid differentiation caused by a prototypical PIEZO1 GoF mutation in DHS. Treatment with mitapivat fully rescued the observed erythropoiesis defects of PIEZO1-KI cells *in vitro*, suggesting the potential benefits of modulating glycolysis with PK activation in models of DHS.

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