Fetal hemoglobin rescues ineffective erythropoiesis in sickle cell disease

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Received: July 1, 2020

Accepted: August 17, 2020.

Pre-published: August 27, 2020.

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Supplemental Methods

Antibodies and fluorescent dies

BV421-conjugated anti-glycophorin A (GPA), APC-conjugated anti-CD49d, FITC- or PE-conjugated anti-HbF, APC-conjugated CD36 mouse monoclonal antibodies were obtained from BD Biosciences. The PE-conjugated anti-Band 3 mouse monoclonal antibody (PE-BRIC6) was obtained from Bristol Institute for Transfusion Sciences. FITC-conjugated anti-HSP70, anti-Lamin A/C and anti-α hemoglobin mouse monoclonal antibodies were obtained from Santa Cruz Biotechnology. Hoechst33342 was obtained from Life Technologies; Hoechst34580, Fixable viability stain 780 (FVS), 7-Aminoactinomycin D (7AAD) and PE-conjugated Annexin V were obtained from BD Biosciences. Rabbit anti-Hsp70/Hsp72 polyclonal antibody was obtained from Enzo Lifesciences. Mouse HRP anti-actin monoclonal antibody was obtained from Santa Cruz Biotechnology. Anti-rabbit and anti-mouse IgG, HRP-linked secondary antibodies were purchased from Cell Signaling. Goat anti-rabbit Alexa 633 and Alexa 488 secondary antibodies were obtained from Invitrogen. DAPI Fluoromount – G mounting media was obtained from Southern Biotech.

In vitro differentiation of human erythroid progenitors

$CD34^+$ cells were placed in an *in vitro* two-phase liquid culture system, as previously described[10]. During the first phase, cells were expanded for 7 days in a medium containing 100 ng/ml of human recombinant (hr) interleukin (IL)-6 (Miltenyi Biotec), 10 ng/ml of hr IL-3 (Miltenyl Biotec) and 50 ng/ml hr stem cell factor (SCF) (Miltenyi Biotec) in Iscove's Modified Dulbecco's Medium IMDM (Gibco) supplemented with 15% BIT 9500 (Stem Cell Technologies), 100 U/ml Penicillin Streptomycin (Gibco) and 2 mM L-Glutamine (Gibco). On day 7, cells were harvested and cultured for 11 days with the second phase medium [10 ng/ml hr IL-3, 50 ng/ml hr SCF, and 2 U/ml of erythropoietin (EPO)]. At day 3 of phase II,

cell suspensions were split into 2 and one half was cultured under partial hypoxia (5% O_2) until day 11 while the other was cultured at normoxia. Cells were diluted at $0.5x10^6$ cells/ml at day 3, 5, 7, 9 and 11 of phase II.

Imaging Flow Cytometry analysis of human bone marrow samples

Bone marrow samples were processed as previously described[14]. For bone marrow samples from SCD patients, mononuclear cells were fixed with PBS 1% formaldehyde (Sigma), 0.025% glutaraldehyde (Sigma) for 15 min, washed twice with PBS, permeabilized with 0.1 M Octyl β-D-Glucopyranoside (Sigma) for 15 min and saturated in PBS, 1% BSA, 2% goat serum for 30 min. Cells were then stained for GPA, CD49d, Band 3 and HbF for 30 min in the dark. Cells stained with the isotype control antibodies were used as a negative control. Compensation was performed using BD Biosciences compensation beads following the supplier instructions. Samples were then analyzed using the Imagestream ISX MkII flow cytometer (Amnis Corp, EMD Millipore) and the INSPIRE software version 99.4.437.0. Acquired data was analyzed using IDEAS software version 6.2 (Amnis Corp, EMD Millipore).

Surface marker staining

In vitro cultured cells were stained for surface expression of GPA, Band 3 and CD49d at day 3, 5, 7, 9 and 11. Briefly, 10^5 cells were suspended in 20 µl of PBS supplemented with 0.5% of bovine serum albumin (BSA), incubated with fluorescence-conjugated antibodies for 30 min in the dark, washed twice with PBS, 0.5% BSA before incubation with 7AAD for 5 min prior to analysis.

Enucleation analysis

Cells (10^5) were washed once with PBS, stained with 1 µg/ml Hoechst34580 for 45 min at 37°C, washed twice with PBS, 0.5% BSA and incubated with anti-CD36 antibody for 30 min in the dark. Cells were then washed twice with PBS, 0.5% BSA and analyzed.

Apoptotic cells

Cells were stained with PE-conjugated Annexin V to measure the percentage of apoptotic cells. Briefly, 10^5 cells were washed once with Annexin buffer, resuspended in 25 µl of the same buffer and stained with 2.5 µl of PE-conjugated Annexin V for 20 min in the dark. Samples were then diluted with 200 µl of Annexin buffer prior to analysis.

HbF and HSP70 staining

Cells were stained with a fixable viability stain (FVS) at a dilution of 1/1000 for 20 min in the dark, washed twice with PBS then fixed with 1% formaldehyde, 0.025% glutaraldehyde in PBS for 15 min. Cells were then washed twice with PBS, permeabilized in 0.1 M Octyl β-D-Glucopyranoside for 15 min and saturated in PBS, 1% BSA, 2% goat serum for 30 min. Cells were then stained with anti-HbF and/or anti-HSP70 and anti-GPA antibodies for 30 min in the dark, washed twice and analyzed. Hoechst33342 nucleus dye was added before analyzing the cells with imaging flow cytometry.

Cytospin

Cytospin was performed using 100,000 cells. Cells were washed twice with PBS and spun on slides using the Cytospin 2 centrifuge (Shandon). Slides were stained with May-Grunwald-Giemsa (MGG) following manufacturer's instructions (Sigma). Slides were then washed with deionized water and left to dry. Slides were covered by a coverslip using the EUKITT classic (O. Kindler ORSA Tech). Cells were imaged using an inverted microscope (Leica DM6000 B) with a 20x/0.4 HCX PL FLUOTAR, equipped with a DFC300 FX color camera. Analysis was performed using the Fiji software[15].

Confocal Microscopy and Proximity Ligation Assay

Cells (10^5) from day 7 of phase II were washed and fixed with 1% formaldehyde, 0.025% glutaraldehyde for 15 min, treated with 50 mM NH_4Cl (Sigma) for 10 min and washed twice with PBS. Cells were then permeabilized with 1% β-D-glucopyranoside (Sigma) for 15 min,

incubated with a saturation solution (1% BSA and 2% goat serum) overnight then with rabbit anti-Hsp70/72 and mouse anti-α hemoglobin (sc-514378 Santa Cruz Biotechnology) for 1 hour at room temperature. After 3 washes with PBS, cells were incubated with goat anti-rabbit Alexa 633 and goat anti-mouse Alexa 488 for 45 min at room temperature, or the PLA secondary antibodies (see below). After a final washing step, cells were spun onto slides and mounted with DAPI. Acquisition was made on LSM700 Zeiss confocal microscope using Zen software. Analysis was performed using Fiji[15]. Fields were selected with respect to cell size, as the smallest cells are the most differentiated. DAPI positive staining was used to define the nuclear region of interest (ROI) and α-globin staining was used to identify the cytoplasmic region. ROI masks were then applied using image calculator to eliminate the nuclear region. Colocalization was quantified using JACoP[23]plugin to calculate the Pearson's correlation coefficient. Thirty cells were analyzed per experiment.

Proximity ligation assay was performed using the Duolink flow PLA Detection Kit – FarRed (Sigma). Cells were incubated with oligonucleotide-conjugated secondary antibodies (PLA probe PLUS anti-mouse and PLA probe MINUS anti-rabbit) for 1 hour at room temperature. Ligation and amplification steps were performed according to the manufacturer's guidelines. For imaging flow cytometry, all incubations were performed with cells in Eppendorf tubes.

Sickling Assay

SCD differentiated erythroblasts at D9 of Phase II of culture were exposed to a 3-stage gradual hypoxia: 15% - 10% - 5% O_2, lasting 20 min each. The time course of sickling was monitored in real time by video microscopy, capturing images every minute throughout the assay, using the AxioObserver Z1 microscope (Zeiss) and a 40x objective.

Supplemental Table 1: X and Y coordinates defining the gating of the Basophilic, Polychromatic and Orthochromatic erythroblasts using imaging flow cytometry. Adapted from a previously described analysis performed on mouse cells[22].

Gate	X-coordinates	Y-coordinates
Basophilic E	54.59	80.709
	90.572	35.092
	121.826	34.949
	145.201	59.226
	148.352	83.891
	143.625	110.412
	135.22	128.976
	109.219	133.22
	87.945	123.407
	73.5	115.716
	59.055	93.704
Polychromatic E	53.802	78.322
	90.046	34.297
	71.398	27.402
	36.993	74.078
Orthochromatic E	35.942	73.813
	22.81	66.918
	18.608	45.436
	31.447	18.915
	53.539	15.467
	69.823	26.341

Video 1: Two SCD erythroblasts at D9 of phase II of culture undergoing gradual hypoxia: 15%, 10% and 5% oxygen. Each partial hypoxia step has a duration of 20 min, with 1 image taken every minute.

Figure S1

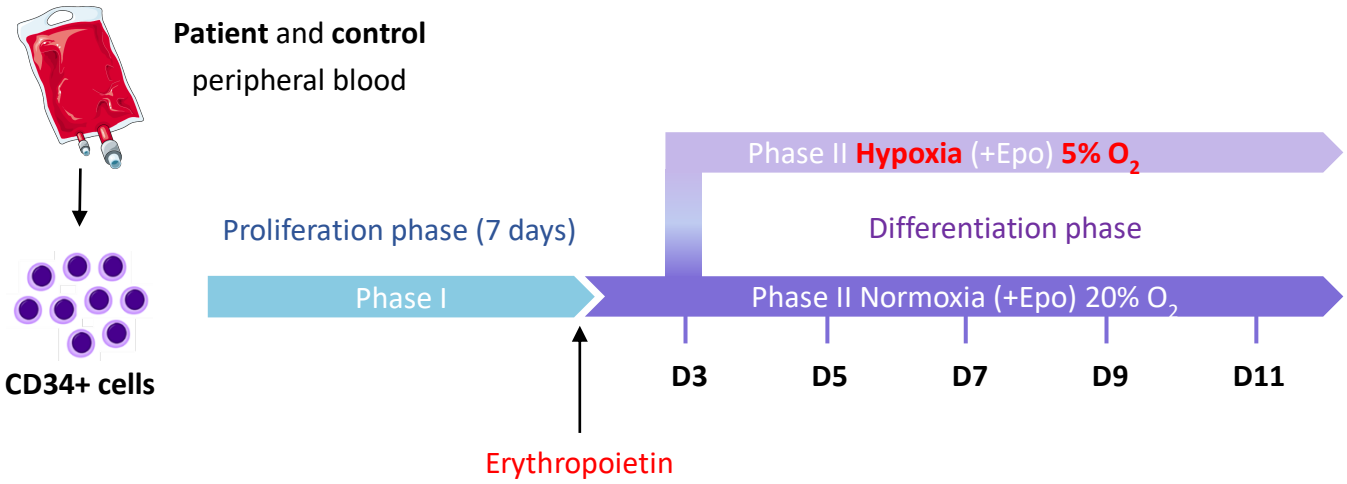

Figure S1: A scheme representing the *in vitro* erythropoiesis protocol. Cells were put under normoxia or partial hypoxia (5% O_2) starting from day 3 (D3) of phase II of culture.

Figure S2

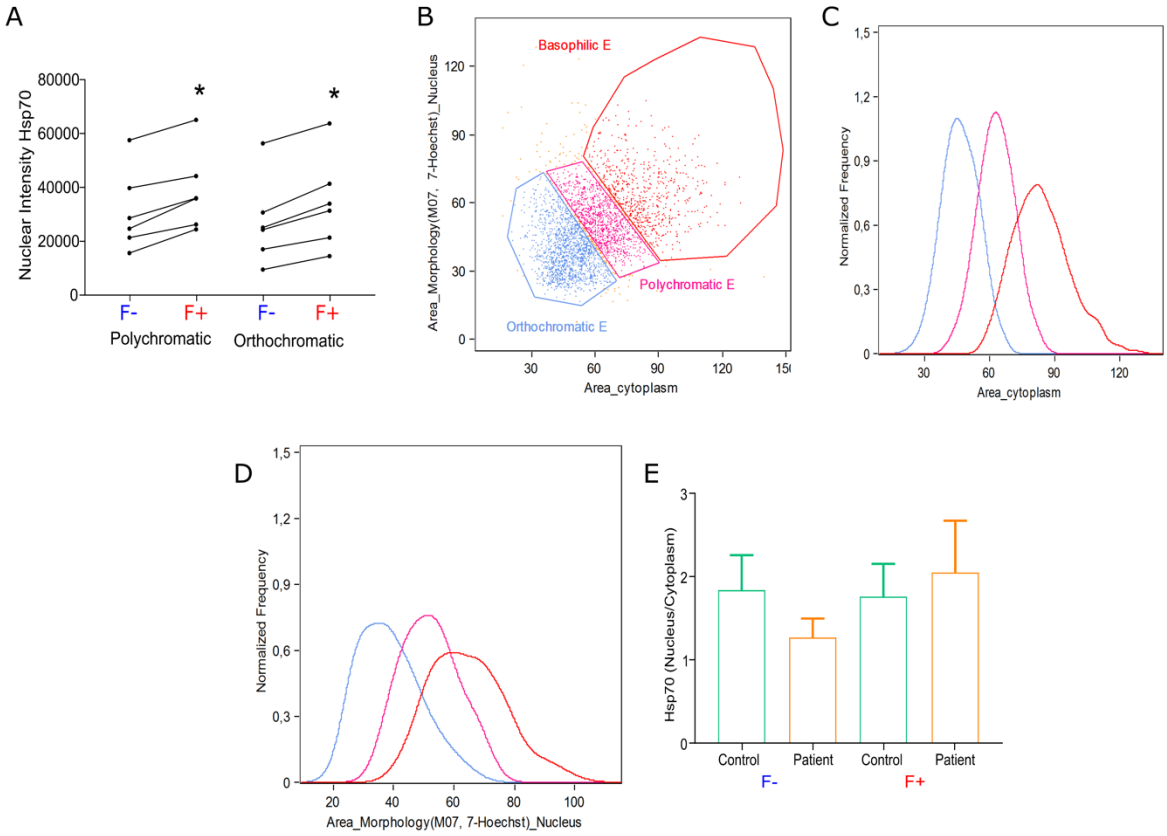

Figure S2: (A) Nuclear intensity of HSP70 in F- and F+ SCD polychromatic and orthochromatic erythroblasts at D7 of phase II of culture under hypoxia (n = 6). (B) Dot plot representing the area of the cytoplasm (x axis) and the area of the nucleus (y axis) of GPA-positive erythroblasts, with gating of basophilic, polychromatic and orthochromatic erythroblasts. Histograms representing the area of the cytoplasm (C) and of the nucleus (D) for the 3 subpopulations (basophilic: red, polychromatic: pink and orthochromatic: blue). (E) HSP70 nucleus/cytoplasm ratio (means ± SEM) in F- and F+ orthochromatic cells of control (n = 3) and SCD samples (n = 6) at D7 of phase II of culture under hypoxia. *$p < 0.05$. Wilcoxon paired test (A).

Figure S3

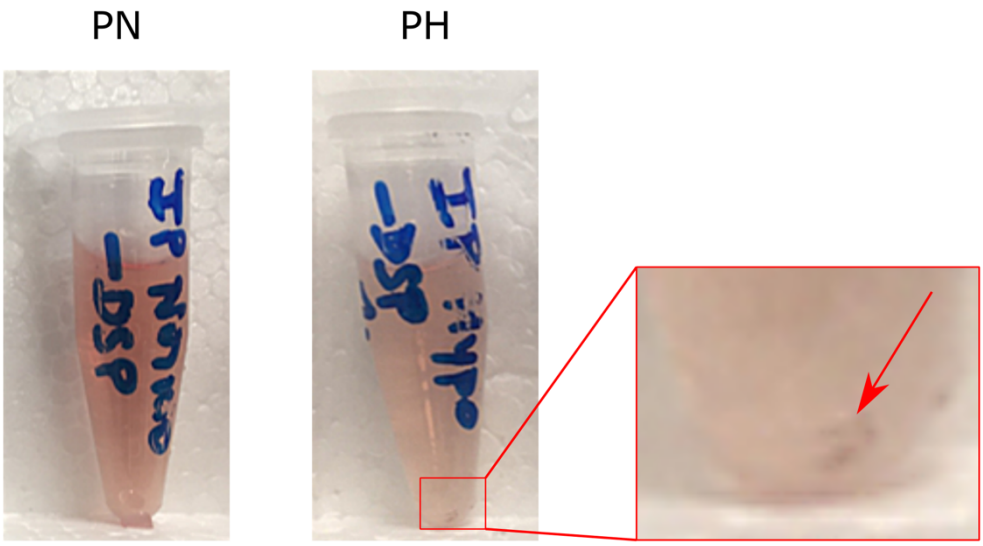

Figure S3: Lysates of identical amounts of SCD RBCs after incubation at normoxia (PN) and hypoxia (PH) and prior to HSP70 immunoprecipitation. The PH lysate is paler, with the presence of a small red colored precipitate as indicated by the arrow in the image at the right.

Figure S4

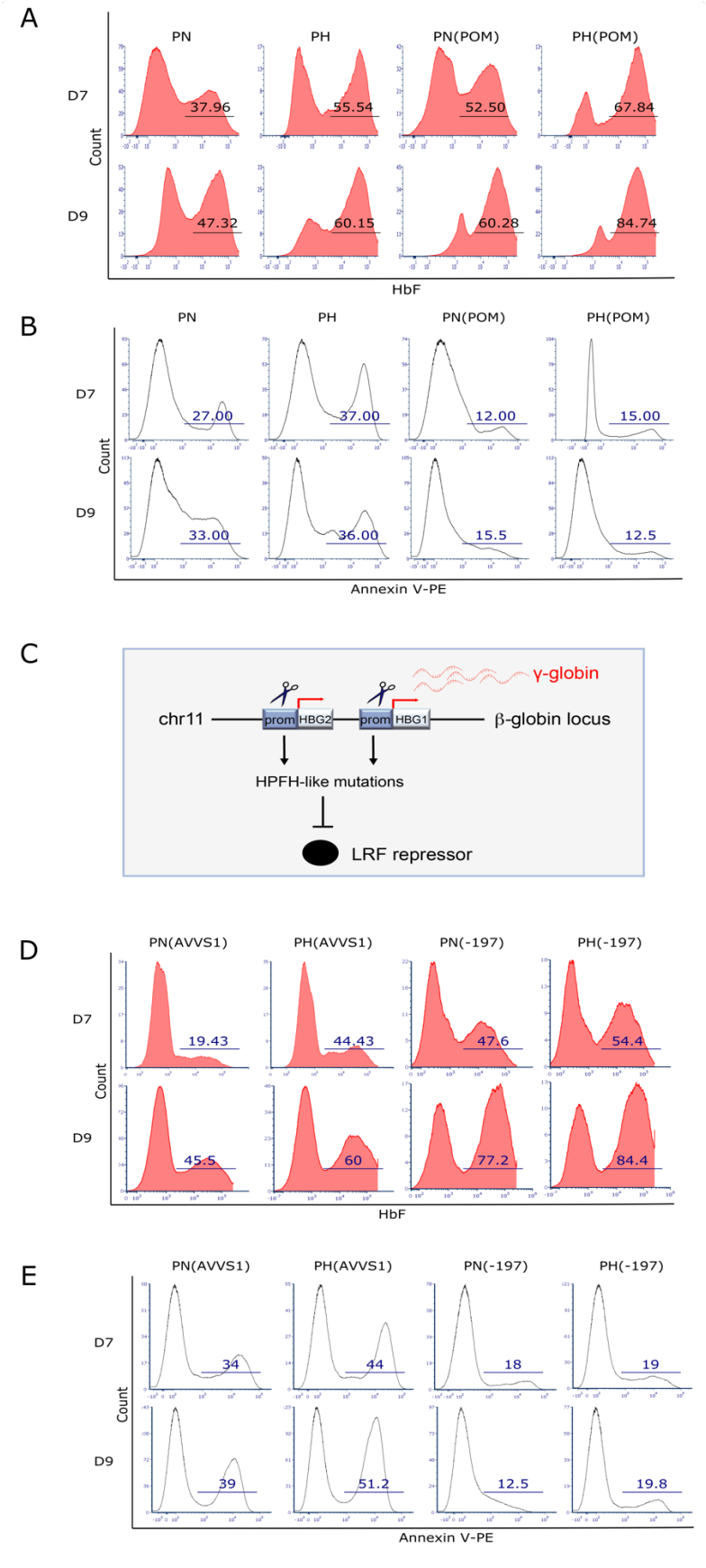
A
PN
PH
PN(POM)
PH(POM)
D7
D9
Count
37.96
55.54
52.50
67.84
47.32
60.15
60.28
84.74
HbF
B
PN
PH
PN(POM)
PH(POM)
D7
D9
Count
27.00
37.00
12.00
15.00
33.00
36.00
15.5
12.5
Annexin V-PE
C
γ-globin
chr11
prom
HBG2
prom
HBG1
β-globin locus
HPFH-like mutations
LRF repressor
D
PN(AVVS1)
PH(AVVS1)
PN(-197)
PH(-197)
D7
D9
Count
19.43
44.43
47.6
54.4
45.5
60
77.2
84.4
HbF
E
PN(AVVS1)
PH(AVVS1)
PN(-197)
PH(-197)
D7
D9
Count
34
44
18
19
39
51.2
12.5
19.8
Annexin V-PE

Figure S4: Flow cytometry plots showing the percentage of (A) F-cells and (B) Annexin V-positive cells at D7 and D9 of phase II of culture in SCD patient erythroblasts under normoxia (PN), hypoxia (PH), normoxia with POM [PN(POM)] and hypoxia with POM [PH(POM)]. (C) Scheme of the CRISPR/Cas9 strategy targeting *HBG1* and *HBG2* γ-globin promoters. Flow cytometry plots showing the percentage of (D) F-cells and (E) Annexin V-positive cells at D7 and D9 of phase II of culture in SCD patient erythroblasts under normoxia (PN) and hypoxia (PH) treated with gRNA targeting the LRF binding site (-197) or an unrelated locus as control (AAVS1).