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RESEARCH PAPER

Pharmacologic induction of PGC-1α stimulates fetal haemoglobin gene expression

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Summary

Sickle cell disease (SCD) is a genetic disorder that affects millions around the world. Enhancement of fetal γ-globin levels and fetal haemoglobin (HbF) production in SCD patients leads to diminished severity of many clinical features of the disease. We recently identified the transcriptional co-activator PGC-1α as a new protein involved in the regulation of the globin genes. Here, we report that upregulation of PGC-1α by infection with a lentivirus expressing PGC-1α or by the small-molecule PGC-1α agonist ZLN005 in human primary erythroid progenitor $CD34^+$ cells induces both fetal γ-globin mRNA and protein expression as well as the percentage of HbF-positive cell (F cells) without significantly affecting cell proliferation and differentiation. We further found that the combination of ZLN005 and hydroxyurea (hydroxycarbamide) exhibited an additive effect on the expression of γ-globin and the generation of F cells from cultured $CD34^+$ cells. In addition, ZLN005 induced robust expression of the murine embryonic βh1-globin gene and to a lesser extent, human γ-globin gene expression in sickle mice. These findings suggest that activation of PGC-1α by ZLN005 might provide a new path for modulating HbF levels with potential therapeutic benefit in β-hemoglobinopathies.

KEYWORDS
human primary erythroid progenitors, PGC-1α, sickle cell disease, ZLN005

INTRODUCTION

Sickle cell disease (SCD) is a common inherited blood disorder caused by a single missense mutation in the β-globin gene. This mutation leads to the synthesis of sickle haemoglobin (HbS), which polymerizes when deoxygenated, damaging the red blood cell (RBC) and causing haemolytic anaemia, vaso-occlusion, pain, progressive multi-organ damage, and in severe cases results in early mortality.[1–3] Around 100 000 people in the United States and more than 3.2 million people globally live with SCD and about 176 000 individuals die yearly from its complications.[4] Giving the compelling biochemical and clinical evidence that increasing fetal haemoglobin (HbF) levels both replaces HbS and inhibits its polymerization,[4–6] therapeutic agents that induce HbF production are expected to benefit patients with SCD.

Hydroxyurea (hydroxycarbamide; HU) has become the standard of care for treating SCD and its beneficial effects are due primarily to its ability to increase HbF.[7] However, the response to HU among patients is variable, and patients who

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respond well to HU can still have life-threatening complications.[5,8,9] Even with HU-induced HbF levels of 20%, some F cells (RBC containing HbF) do not have sufficient HbF to prevent polymerization-induced damage.[5] Therefore, additional pharmacological agents that increase HbF levels while broadening its distribution in F cells are highly desired, particularly those that activate HbF synthesis by targeting different pathways and might be utilized in combination with currently available therapeutics.

The peroxisome proliferator-activated receptor-γ coactivator-1α (PPARGC1A or PGC-1α) is the first member of the PGC-1 family of coactivators and was initially discovered in brown fat as a PPARγ-interacting protein.[10] PGC-1α plays a major role in cellular energy metabolism, mitochondrial oxidative metabolism and lipid and glucose homeostasis.[10–12] Moreover, PGC-1α has a strong transcriptional action when linked to a heterologous DNA-binding domain and when binding to transcription factors that can displace repressor proteins such as histone deacetylase (HDAC) on its target promoters leading to enhanced gene transcription.[10,13,14] We recently found PGC-1α to be involved in the regulation of globin genes.[15] Loss-of-function PGC-1α-knockout mice displayed reduced expression of embryonic εy- and βh1-globin genes.[15] Forced overexpression of PGC-1α *in vitro* by adenovirus infection in bone-marrow cells from SCD mice resulted in significantly increased expression of human γ-globin (>sixfold) and murine εy-globin (fourfold) and βh1-globin (eightfold) genes.[16] These results suggested that modulating PGC-1α activity or the signalling pathways that it regulates might therapeutically benefit patients with SCD.

We show herein that PGC-1α overexpression in human primary erythroid progenitor $CD34^+$ cells increased the expression of γ-globin at both mRNA and protein levels and the number of F cells. A small-molecule agonist of PGC-1α, ZLN005, could effectively activate the expression of PGC-1α leading to the induction of γ-globin genes and HbF synthesis in cultured $CD34^+$ cells. Notably, the combination of ZLN005 and HU had an additive effect, resulting in significantly more F cells in cultured $CD34^+$ cells. Moreover, ZLN005-treated sickle mice exhibited slightly increased γ-globin mRNA expression but markedly elevated murine endogenous embryonic βh1-globin mRNA expression. This study constitutes firm evidence that PGC-1α is a novel molecular target for HbF induction and that ZLN005 exerts potential therapeutic effects for treating SCD.

METHODS

Human $CD34^+$ cell purification and culture

Human $CD34^+$ cells were purified from peripheral blood of de-identified normal volunteers using anti-$CD34^+$ antibody-conjugated microbeads and Mini-MACS column according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). Purified $CD34^+$ cells were cultured in a three-phase culture system plus an expansion phase. For the expansion phase, $CD34^+$ cells were expanded in StemSpan™ SFEM II media (StemCell Technologies, Vancouver, BC, Canada) supplemented with StemSpan™ $CD34^+$ Expansion Supplement for five days at a density of 1×10^5 cells/ml. After expansion, $CD34^+$ cells were put in erythroid differentiation medium (EDM) which consists of Iscove's Modified Dulbecco's Medium with 1% L-glutamine, 2% penicillin–streptomycin, 330 μg/ml holo-human transferrin, 10 μg/ml recombinant human insulin, 2 IU/ml heparin, 5% heat-inactivated human peripheral blood plasma, and 3 IU/ml erythropoietin. For phase 1 (Day 0–7), EDM was supplemented with 10^{-5} M hydrocortisone, 100 ng/ml human stem cell factor (hSCF), and 5 ng/ml IL-3. At phase 2 (Day 7–11), EMD was supplemented with 100 ng/ml hSCF. Then, at phase 3 (Day 11–18), EMD was used without any additional supplement. Cells were maintained at a density of 2×10^5 cells/ml for phases 1 and 2, or 0.5 to 1×10^6 cells/ml for phase 3.

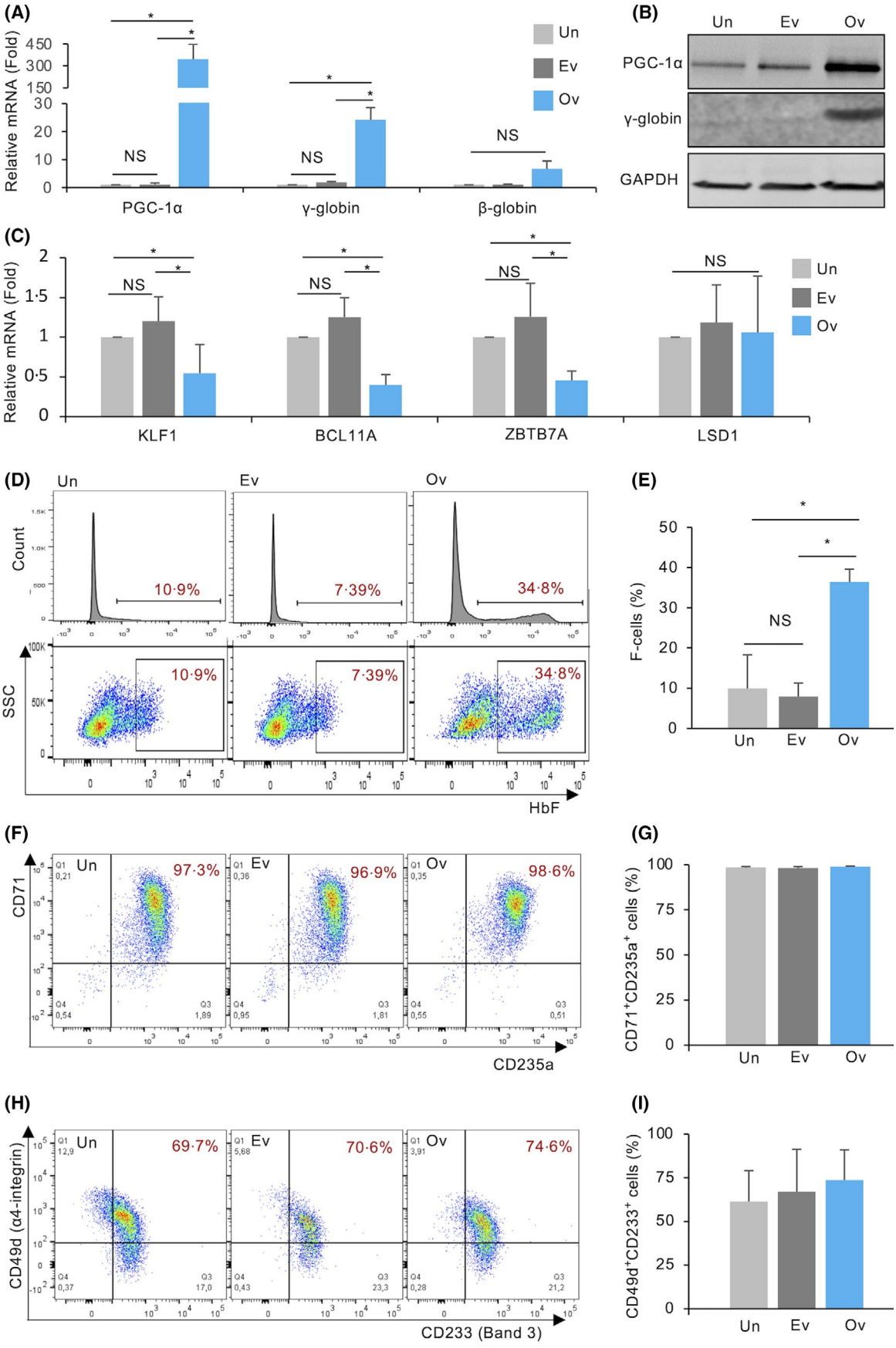
Human umbilical cord-derived erythroid progenitors (HUDEP-1)

HUDEP-1 cells were cultured in a proliferation medium consisting of StemSpan™ SFEM I with 50 ng/ml hSCF, 1 μM dexamethasone, 1 μg/ml doxycycline, 3 U/ml erythropoietin and 1% L-glutamine.[17]

Sickle cell disease mice

Six- to 8-week-old SCD mice (hα/hα:β^S/β^S, Townes model) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animal experiments were approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine.

FIGURE 1 PGC-1α overexpression in human primary $CD34^+$ cells. (A) Relative mRNA abundance of *PGC-1α* and globin genes in $CD34^+$ cells infected with lentivirus. (B) Western blot analysis of PGC-1α and γ-globin protein expression. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as a loading control. (C) Relative mRNA expression level of some haemoglobin regulators in $CD34^+$ cells upon PGC-1α overexpression. (D) Representative flow-cytometric profiles and histograms of F cell percentage in cultured $CD34^+$ cells gated from CD235a-positive populations. (E) A summary of five independent experiments showing a significant increase of F cells in PGC-1α-overexpressed cells. (F) Representative flow-cytometric profiles of cell differentiation markers CD71 and CD235a in infected $CD34^+$ cells. (G) Statistical analysis of the percentage of $CD71^+CD235a^+$ double-positive cells by flow cytometry averaged over all samples. (H) Representative flow-cytometric profile of terminal differentiation markers CD49d and CD233 in infected $CD34^+$ cells. (I) Statistical analysis of the percentage of $CD49d^+CD233^+$ double-positive cells by flow cytometry averaged over all samples. Data are presented as mean ± SD, $n = 5$ independent experiments. Statistically significant differences between PGC-1α-overexpressed and control cells are indicated (*, $p < 0.05$). Ev, empty vector; NS, no statistical significance; Ov, PGC-1α overexpression; Un, untreated [Colour figure can be viewed at wileyonlinelibrary.com]


(A)
Relative mRNA (Fold)
450
300
150
30
20
10
0
NS
NS
NS
*
*
*
*
Un
Ev
Ov
PGC-1α
γ-globin
β-globin
(B)
Un
Ev
Ov
PGC-1α
γ-globin
GAPDH
(C)
Relative mRNA (Fold)
2
1·5
1
0·5
0
NS
NS
NS
NS
*
*
*
*
Un
Ev
Ov
KLF1
BCL11A
ZBTB7A
LSD1
(D)
Un
Ev
Ov
Count
10·9%
7·39%
34·8%
SSC
100K
50K
0
10·9%
7·39%
34·8%
HbF
(E)
F-cells (%)
50
40
30
20
10
0
NS
*
*
Un
Ev
Ov
(F)
CD71
Un
Ev
Ov
97·3%
96·9%
98·6%
Q1 0,21
Q4 0,54
Q3 1,89
Q1 0,36
Q4 0,95
Q3 1,81
Q1 0,35
Q4 0,55
Q3 0,51
CD235a
(G)
CD71+CD235a+ cells (%)
100
75
50
25
0
Un
Ev
Ov
(H)
CD49d (α4-integrin)
Un
Ev
Ov
69·7%
70·6%
74·6%
Q1 12,9
Q4 0,37
Q3 17,0
Q1 5,68
Q4 0,43
Q3 23,3
Q1 3,91
Q4 0,28
Q3 21,2
CD233 (Band 3)
(I)
CD49d+CD233+ cells (%)
100
75
50
25
0
Un
Ev
Ov

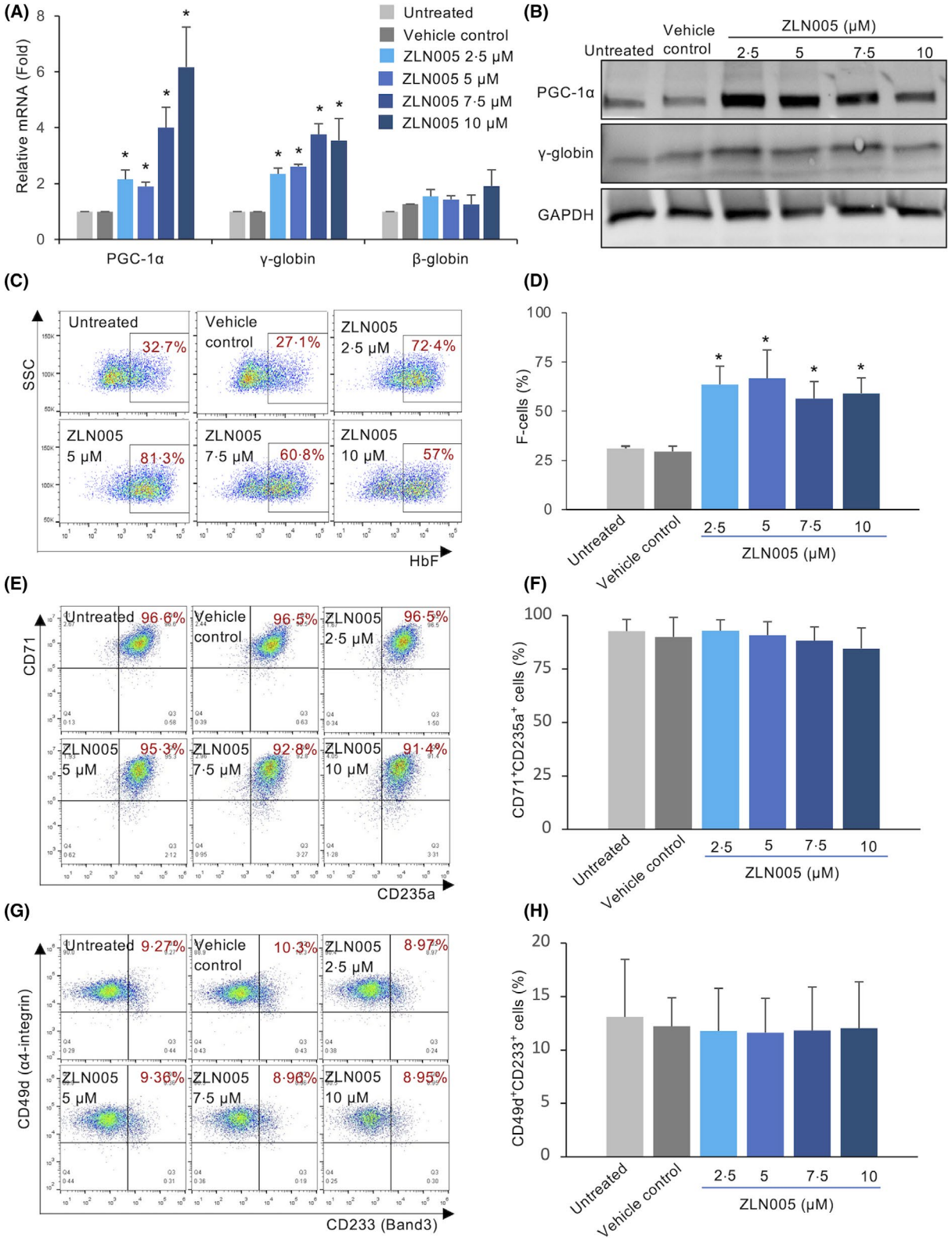


FIGURE 2 Effects of ZLN005 on HUDEP-1 cells. (A) Relative mRNA abundance of PGC-1α and globin genes in HUDEP-1 cells treated with ZLN005 or vehicle control (DMSO) for 2 days. (B) Western blots depict the abundant expression of PGC-1α and γ-globin in treated HUDEP-1 cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as a loading control. (C) Representative flow-cytometric profiles of treated HUDEP-1 cells after HbF staining. (D) Statistical analysis of the percentage of F cells by flow cytometry averaged over all samples. (E, G) Representative flow-cytometric profiles of CD71/CD235a or CD49d/CD233 in treated HUDEP-1 cells. (F, H) Statistical analysis of the percentage of $CD71^+CD235a^+$ or $CD49d^+CD233^+$ cells averaged over all samples. Data are presented as mean ± SD, $n = 3$ independent experiments. Statistically significant differences between treated and control cells are indicated (*, $p < 0.05$) [Colour figure can be viewed at wileyonlinelibrary.com]

Lentivirus production and transduction

For PGC-1α overexpression, *PGC-1α* cDNA was subcloned into the pLVX-TetOne-Puro vector (Takara Bio) and driven by the TRE3GS promoter. The vector has a puromycin-resistant gene which is driven by the SV40 promoter. Lenti-X 293 T cells (Takara Bio, Otsu, Shiga, Japan) were cultured in a 10 cM collagen-coated dish (Corning Life Sciences, Corning, NY, USA) with Dulbecco's Modified Eagle Medium supplemented with 20% fetal bovine serum, 1% glutamine and 1% penicillin–streptomycin. When cells reached 80%–90% confluency, they were transfected with lentivirus vectors using Lenti-X-packaging single shots. The supernatants were harvested at 48 h and 72 h after transfection. Lentivirus particles in the supernatant were concentrated using a Lenti-X concentrator.

$CD34^+$ cells were seeded to 96-well plates at a density of 4×10^4 cells/well in phase-1 medium. Lentivirus transductions were carried out on Day 1 at the multiplicity of infection (MOI) 50 with 5 μg/ml polybrene. Cells were incubated in 37°C for 4–6 h and transferred to fresh phase-1 medium in six-well plates. On Day 4 of phase 1, puromycin (2 μg/ml) and doxycycline (1 μg/ml) were added into the medium for the selection of infected cells and inducing of PGC-1α overexpression. After 7 days of selection, cells were collected for further analyses.

Flow-cytometric analysis

F cells were quantified by flow cytometry after lentivirus infection or drug exposure for $CD34^+$ and HUDEP-1. Cells were fixed with 0.08% glutaraldehyde in phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100, and then stained with 5 μl Allophycocyanin (APC)-conjugated HbF antibody (Thermo Fisher Scientific, Waltham, MA, USA) or APC anti-human IgG1 antibody (BioLegend, San Diego, CA, USA) for 15 min in the dark at room temperature. Flow-cytometric analyses were performed on LSRII (BD Biosciences, San Jose, CA, USA), and data were analysed using FlowJo software.

Quantitative real-time polymerase chain reaction

Globin gene mRNA abundance was determined by the quantitative real-time polymerase chain reaction (qRT-PCR). Primers for qRT-PCR are listed in Table S1. TaqMan primers (Thermo Fisher Scientific, Waltham, MA, USA) were used to determine the mRNA levels of the *KLF1*, *BCL11A*, *ZBTB7A*, and *LSD1* genes. It should be noted that *OAZ1* was used as a housekeeping gene control.[18]

Western blot analysis

Cell lysis was prepared using radioimmunoprecipitation assay (RIPA) buffer with fresh proteinase inhibitors (Sigma Aldrich, St Louis, MO, USA). Samples were loaded to a 4%–20% mini-PROTEAN TGX Gel (Bio-Rad, Hercules, CA, USA) and separated by electrophoresis, and transferred to a nitrocellulose membrane. After blocking with 3% bovine serum albumin (BSA) for 1 h at room temperature, the membrane was incubated with antibodies against PGC-1α (Novus Biologicals, Littleton, CO, USA), HbF-FITC (Thermo Fisher Scientific), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) overnight at 4°C, and then followed with fluorescent secondary antibody incubation (Li-Cor). The images were acquired with iBright FL1500 (Thermo Fisher Scientific).

Chromatin immunoprecipitation assays

HUDEP-1 cells were fixed with 2 mM ethylene glycol-bis succinimidylsuccinate (Thermo Fisher Scientific) at room temperature for 30 min before the addition of 1% formaldehyde as described previously.[19] After incubating chromatin of 1×10^7 cells with 5 μg PGC-1α or control IgG antibodies, co-precipitated DNA was purified and subjected to qPCR assay to quantify recovery of the globin gene promoter regions. The abundance of immunoprecipitated DNAs relative to input DNA, and relative to control IgG, were determined to evaluate the specific association of PGC-1α proteins with the globin gene promoters. Primers for chromatin immunoprecipitation (ChIP) assays are listed in Table S2.

Drug preparation

ZLN005 was purchased from Cayman Chemical (Ann Arbor, MI, USA). It was first dissolved in dimethylsulphoxide (DMSO) at the concentration of 20 mM stock and then diluted in DMSO to the desired working solution.

RESULTS

Effects of PGC-1α overexpression on haemoglobin and erythroid differentiation

To explore the role of PGC-1α in γ-globin regulation, we examined the effect of PGC-1α overexpression on γ-globin synthesis. We infected human primary erythroid progenitor $CD34^+$ cells with a lentivirus carrying *PGC-1α* full-length cDNA (pLVX-TetOne-Puro-PGC-1α). Moreover, the lentivirus generated with the empty vector (pLVX-TetOne-Puro) was used as a control. Infection with the PGC-1α overexpression virus significantly increased the abundance of *PGC-1α* mRNA (>300-fold) and protein compared with the empty vector control virus (Figure 1A,B). PGC-1α overexpression resulted in enhanced γ-globin mRNA synthesis that was increased nearly 20-fold compared with controls, while the adult β-globin mRNA levels were not increased significantly (Figure 1A). The increases of γ-globin protein expression in PGC-1α-overexpressing cells were confirmed by Western blot analysis (Figure 1B). Additionally, the levels of major globin

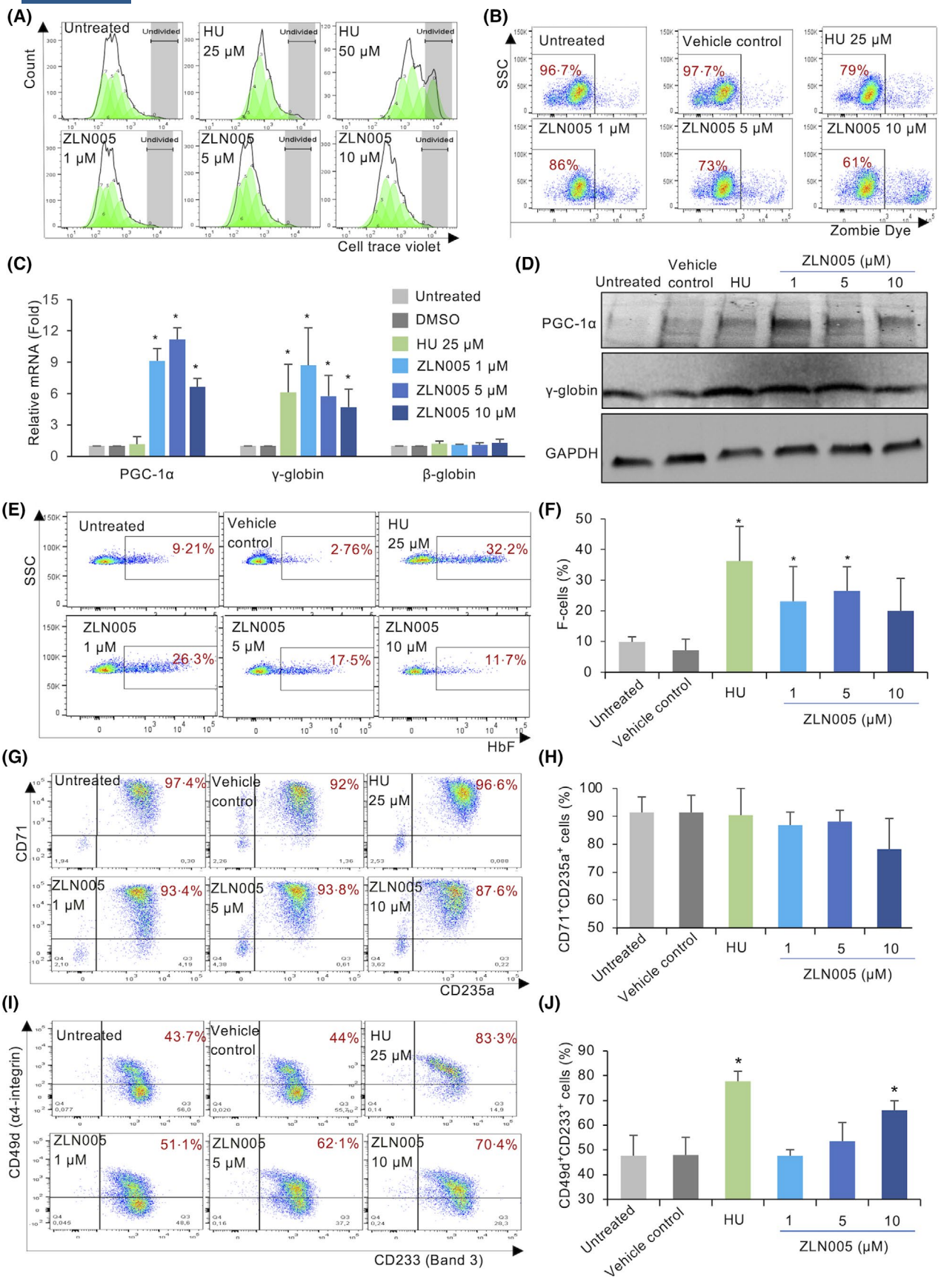

(A)
Untreated
HU 25 μM
HU 50 μM
ZLN005 1 μM
ZLN005 5 μM
ZLN005 10 μM
Undivided
Count
Cell trace violet
(B)
Untreated
Vehicle control
HU 25 μM
ZLN005 1 μM
ZLN005 5 μM
ZLN005 10 μM
96·7%
97·7%
79%
86%
73%
61%
SSC
Zombie Dye
(C)
Relative mRNA (Fold)
Untreated
DMSO
HU 25 μM
ZLN005 1 μM
ZLN005 5 μM
ZLN005 10 μM
PGC-1α
γ-globin
β-globin
(D)
Untreated
Vehicle control
HU
ZLN005 (μM)
1
5
10
PGC-1α
γ-globin
GAPDH
(E)
Untreated
Vehicle control
HU 25 μM
ZLN005 1 μM
ZLN005 5 μM
ZLN005 10 μM
9·21%
2·76%
32·2%
26·3%
17·5%
11·7%
SSC
HbF
(F)
F-cells (%)
Untreated
Vehicle control
HU
ZLN005 (μM)
(G)
Untreated
Vehicle control
HU 25 μM
ZLN005 1 μM
ZLN005 5 μM
ZLN005 10 μM
97·4%
92%
96·6%
93·4%
93·8%
87·6%
CD71
CD235a
(H)
CD71+CD235a+ cells (%)
Untreated
Vehicle control
HU
ZLN005 (μM)
(I)
Untreated
Vehicle control
HU 25 μM
ZLN005 1 μM
ZLN005 5 μM
ZLN005 10 μM
43·7%
44%
83·3%
51·1%
62·1%
70·4%
CD49d (α4-integrin)
CD233 (Band 3)
(J)
CD49d+CD233+ cells (%)
Untreated
Vehicle control
HU
ZLN005 (μM)

FIGURE 3 Effects of ZLN005 on human primary CD34$^+$ cells. (A) Proliferation assay of CD34$^+$ cells treated with ZLN005 or hydroxyurea (HU) for 4 days using cell trace violet proliferation kit. Grey-shadowed populations are undivided (proliferation delayed) cells. (B) Flow-cytometric analysis of the viability of treated CD34$^+$ cells using Zombie dye staining (BioLegend, San Diego, CA, USA). Live cells are Zombie dye-negative populations. (C) Relative mRNA abundance of PGC-1α and γ- and β-globin in treated CD34$^+$ cells. (D) Western blots depict the abundant expression of PGC-1α and γ-globin in treated CD34$^+$ cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as a loading control. (E) Representative flow-cytometric profiles of treated CD34$^+$ cells after HbF staining. (F) Statistical analysis of the percentage of F cells by flow cytometry averaged over all samples. (G, I) Representative flow-cytometric profiles of CD71/CD235a or CD49d/CD233 in treated CD34$^+$ cells. (H, J) Statistical analysis of the percentage of CD71$^+$CD235a$^+$ or CD49d$^+$CD233$^+$ double-positive cells by flow cytometry averaged over all samples. Data are presented as mean ± SD, $n = 5$ independent experiments. Statistically significant differences between treated and control cells are indicated (*, $p < 0.05$) [Colour figure can be viewed at wileyonlinelibrary.com]

regulators such as *BCL11A*,[20,21] *ZBTB7A*[22] and *KLF1*,[23] were reduced in expression upon PGC-1α overexpression, but the mRNA levels of *LSD1*[24] were unaffected (Figure 1C). Flow-cytometric analysis using an anti-HbF antibody showed that the percentage of F cells was 36.5% ± 3.1% in CD34$^+$ cells overexpressing PGC-1α, whereas in untreated or control empty vector lentivirus-infected cells, the F-cell numbers were 10% ± 8.4% or 7.9% ± 3.3%, respectively (Figure 1D,E, also refer to Figure S1 for gating strategies). Consistent with the increase of F-cell numbers, the histograms of F cells showed a clear peak with higher mean fluorescence intensity in PGC-1α-overexpressing cells, suggesting that the overexpression of PGC-1α not only increased the number of F cells but also increased the concentration of HbF in F cells (Figure 1D).

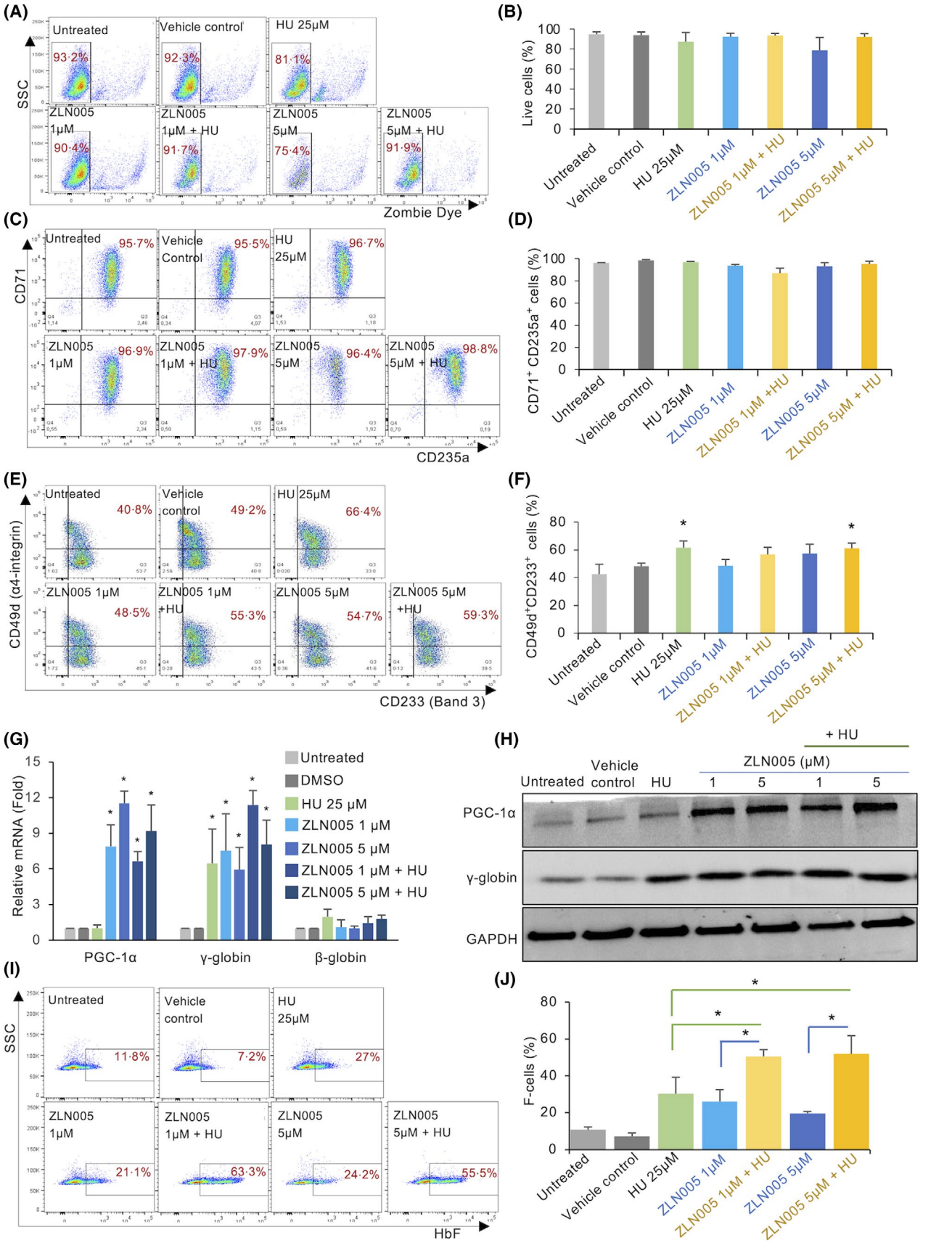
Thereafter, we examined the effect of PGC-1α overexpression on erythroid differentiation by flow-cytometric analyses of lentivirus-infected CD34$^+$ cells stained with antibodies against THE cellular differentiation marker transferrin receptor (CD71) and the erythroid-specific marker CD235a (Figure 1F,G), as well as terminal differentiation markers CD49d (α4-integrin) and CD233 (Band 3) (Figure 1H,I). Most cells expressed both CD71 and CD235a, indicating proper differentiation into the erythroid lineage (Figure 1F). The number of CD71$^+$CD235a$^+$ double-positive cells was similar between PGC-1α-overexpressing cells and control cells, suggesting that PGC-1α overexpression did not affect cell-differentiation status (Figure 1G). Consistently, the other differentiation markers (CD49d/CD233) were not significantly different in PGC-1α-overexpressing and control cells (Figure 1I). These results suggest that PGC-1α might have a role in γ-globin regulation independent of erythroid differentiation.

PGC-1α modulation in HUDEP-1 cells

To develop a new γ-globin inducer based on PGC-1α activation, we used a human erythroid progenitor cell line (HUDEP-1) for quick screening of small molecules that may serve as PGC-1α agonists for γ-globin induction. Undifferentiated HUDEP-1 cells express low levels of γ-globin and almost no β-globin, making them a good cell culture model in screening γ-globin inducers because of the simplicity and reproducibility. A small-molecule compound, ZLN005, was reported to be a selective activator of *PGC-1α* mRNA expression in skeletal muscle cells.[25] To assay the activity of ZLN005 in HUDEP-1 cells, we treated undifferentiated HUDEP-1 cells with various concentrations of ZLN005 for 2 days. ZLN005 administration at all tested doses (from 2.5 to 10 μM) effectively increased *PGC-1α* mRNA expression two- to eightfold compared with untreated or DMSO controls (Figure 2A). The γ-globin mRNA expression was induced more than twofold at 2.5 or 5 μM ZLN005 and nearly fourfold at 7.5 or 10 μM ZLN005, while adult β-globin mRNA levels were almost unaffected (Figure 2A). Protein levels of PGC-1α and γ-globin were determined by Western blots and were increased in HUDEP-1 cells treated with ZLN005 at all tested concentrations. ZLN005 at 2.5 or 5 μM was associated with higher levels of PGC-1α and γ-globin than cells treated with 7.5 or 10 μM ZLN005, possibly because of cytotoxicity occurring at high concentrations (Figure 2B). Consistent with the Western blot results, flow-cytometric analysis of F cells showed >twofold increase of the percentage of F cells in ZLN005-treated HUDEP-1 cells compared with untreated and vehicle controls (Figure 2C, also refer to Figure S1 for gating strategies). In three independent experiments the greatest induction of F cells was achieved at the concentration of 2.5 μM ZLN005 (63.6% ± 9.3%) or 5 μM ZLN005 (66.7% ± 14.4%) compared with untreated (31.1% ± 1.1%) or vehicle control (29.5% ± 2.7%); 7.5 or 10 μM ZLN005 also significantly increased F-cell numbers, by 56.5% ± 8.6% or 59.1% ± 7.9%, respectively (Figure 2D). Furthermore, we examined the effect of ZLN005 on HUDEP-1 cell differentiation by flow-cytometric analyses and observed no significant changes on differentiation markers CD71/CD235a (Figure 2E,F) and CD49d/CD233 (Figure 2G,H) between ZLN005-treated and control HUDEP-1 cells. The majority of cells were still at undifferentiation status evidenced by the low CD233 (Band3) expression (Figure 2G), suggesting that the HbF-inductive effect of ZLN005 is not due to inducing cell differentiation. In addition, the HbF-inducing effect of ZLN005 was validated in another HUDEP cell line (HUDEP-2) which exclusively expresses a high level of adult β-globin with no γ-globin expression.[17] However, HUDEP-2 cells required an extra 3–5 days of ZLN005 treatment to exhibit increased numbers of F cells in the culture (Figure S2).

Effects of ZLN005 on human primary erythroid progenitor CD34$^+$ cells

To examine the effects of ZLN005 on human primary erythroid progenitors, CD34$^+$ cells were cultured in a three-phase



system and treated with ZLN005 or HU at Day 4 of phase 1 culture. Using CellTrace Violet Cell Proliferation Kit (Thermo Fisher Scientific) and flow-cytometric assay after treatment for 4 days, we found that ZLN005 administration at 1, 5, or 10 μM did not alter cell proliferation, while 50 μM HU significantly reduced cell proliferation with more than

FIGURE 4 Effects of ZLN005 in combination with hydroxyurea (HU) on human primary $CD34^+$ cells. (A) Flow-cytometric analysis of the viability of ZLN005-treated $CD34^+$ cells with or without HU. (B) Statistical analysis of the percentage of live cells averaged over all samples. (C, E) Representative flow-cytometric profiles of CD71/CD235a or CD49d/CD233 in treated $CD34^+$ cells. (D, F) Statistical analysis of the percentage of $CD71^+CD235a^+$ or $CD49d^+CD233^+$ double-positive cells by flow cytometry averaged over all samples. (G) Relative mRNA abundance of *PGC-1α* and γ- and β-globin in treated $CD34^+$ cells. (H) Western blots depict the abundant expression of PGC-1α and γ-globin in treated $CD34^+$ cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as a loading control. (I) Representative flow-cytometric profiles of treated $CD34^+$ cells after HbF staining. (J) Statistical analysis of the percentage of F cells by flow cytometry averaged over all samples. Data are presented as mean ± SD, $n = 3$ independent experiments. Statistically significant differences between treated and control cells are indicated (*, $p < 0.05$) [Colour figure can be viewed at wileyonlinelibrary.com]

40% of cells remaining undivided (Figure 3A). Cell viability measured by the Zombie dye staining (BioLegend, San Diego, CA, USA) showed that ZLN005 at doses less than 10 μM did not statistically affect cell viability. At doses of 1 or 5 μM ZLN005, the percentage of live cells was 86% or 73%, respectively, which was comparable to that of 25 μM HU (79%); 10 μM ZLN005 reduced viability to 61% live cells (Figure 3B). Morphological analysis showed that most of cells were erythroblasts at this stage and the percentage of enucleated cells was 1%–5% in both control and treated cells (Figure S3). After 4 days of treatment, *PGC-1α* mRNA expression was enhanced by ZLN005 up to 9.1-fold at 1 μM, 11.2-fold at 5 μM, or 6.7-fold at 10 μM. The mRNA expression of γ-globin was well correlated with *PGC-1α* levels and increased around four- to eightfold, in $CD34^+$ cells treated with 1, 5, or 10 μM of ZLN005, respectively. Adult β-globin transcription was not significantly affected by ZLN005 treatment (Figure 3C). To be noted, after 13 days of culture when cells were undergoing enucleation and maturation (Figure S3), we did not detect any significant changes in globin mRNA expression in either ZLN005- or HU-treated samples. The protein-level increases of PGC-1α and γ-globin were determined by the Western blot analysis (Figure 3D). Flow-cytometric analysis confirmed the HbF induction in treated cells and showed that the number of F cells was increased by ZLN005 at all doses, 1 μM (23.1% ± 6%), 5 μM (26.5% ± 7.9%) or 10 μM (20% ± 10.6%), compared with untreated (9.8% ± 1.7%) or vehicle control (7.1% ± 3.6%). HU as a positive control also showed an increased F-cell percentage (36.2% ± 9.3%) in the culture (Figure 3E,F).

We next determined whether ZLN005 could affect erythroid differentiation by flow cytometry after staining treated cells with antibodies against CD71 and CD235a or CD49d and CD233. ZLN005 treatment for 9 days at all doses had no significant effect on cell differentiation based on CD71 and CD235a markers (Figure 3G,H). However, terminal differentiation measured by CD49d and CD233 expression was significantly affected by 10 μM ZLN005. The total percentage of $CD49d^+$/$CD233^+$ double-positive cells was 66% ± 3.9% compared with 48% ± 7.1% in vehicle controls. HU, known to delay terminal differentiation, was associated with 78% ± 4% $CD49d^+$/$CD233^+$ positivity. Importantly, doses of 1 μM or 5 μM ZLN005 did not significantly affect terminal differentiation (Figure 3I,J).

ZLN005 in combination with HU

To examine whether ZLN005 and HU have an additive or synergistic effect on HbF induction, we studied the effect of combined treatment in $CD34^+$ cells. Thus, 25 μM HU plus 1 or 5 μM ZLN005 did not affect cell viability (Figure 4A,B). The expression of cell differentiation markers CD71/CD235a in all groups was not significantly different (Figure 4C,D). There was a significant delay in terminal differentiation with the combination of 5 μM ZLN005 and 25 μM HU, where the percentage of $CD49d^+CD233^+$ cells significantly increased to 61% ± 3.8% compared with 48% ± 2.3% in vehicle controls (Figure 4E,F). ZLN005 at 1 and 5 μM with 25 μM HU had higher levels of γ-globin mRNA and protein (Figure 4G,H), and increased F-cell numbers two- to threefold compared with HU alone (Figure 4I,J). This effect of ZLN005 and HU on HbF induction supports the suggestion that these compounds may have different mechanisms of action and that their effects might be additive.

Effects of ZLN005 on SCD mice

To investigate the *in vivo* effects of ZLN005 on γ-globin gene expression, we intraperitoneally administered ZLN005 (1 μg/g body weight/day) to SCD mice. In those mice, the human fetal ^{A}γ-globin gene was placed in a position that would mimic its location and orientation between the murine embryonic and human adult β^S-globin genes. Therefore, the inserted human ^{A}γ-globin gene is regulated as murine embryonic β-like globin genes, and the adult SCD mice exhibit low HbF background (~0.2%) in the peripheral blood. After four consecutive weeks of treatment with ZLN005, we quantified the relative mRNA abundance of *PGC-1α* and globin gene expression by qRT-PCR (Figure 5A). The mRNA levels of *PGC-1α* and γ-globin were slightly increased in ZLN005-treated animals compared with vehicle-treated control mice. Notably, the mRNA levels of murine endogenous embryonic βh1-globin were significantly increased up to ninefold after four weeks of ZLN005 treatment. Both adult β-globin and murine εy-globin transcriptions were unaffected by ZLN005 administration (Figure 5A). Flow-cytometric analysis using an anti-HbF antibody showed that the F-cell population was induced about twofold in the peripheral blood of ZLN005-treated SCD mice (Figure 5B,C). These data demonstrated that ZLN005 has the ability to induce *in vivo* the expression of embryonic and fetal globin genes which are normally repressed in adult SCD mice.

We considered the possibility that the 1 400-bp 5′ promoter fragment of the γ-globin gene transgene in SCD mice lacks regulatory elements or binding sites for PGC-1α. To address this, we examined the chromatin occupancy of PGC-1α across the promoter region of the γ-globin gene by ChIP

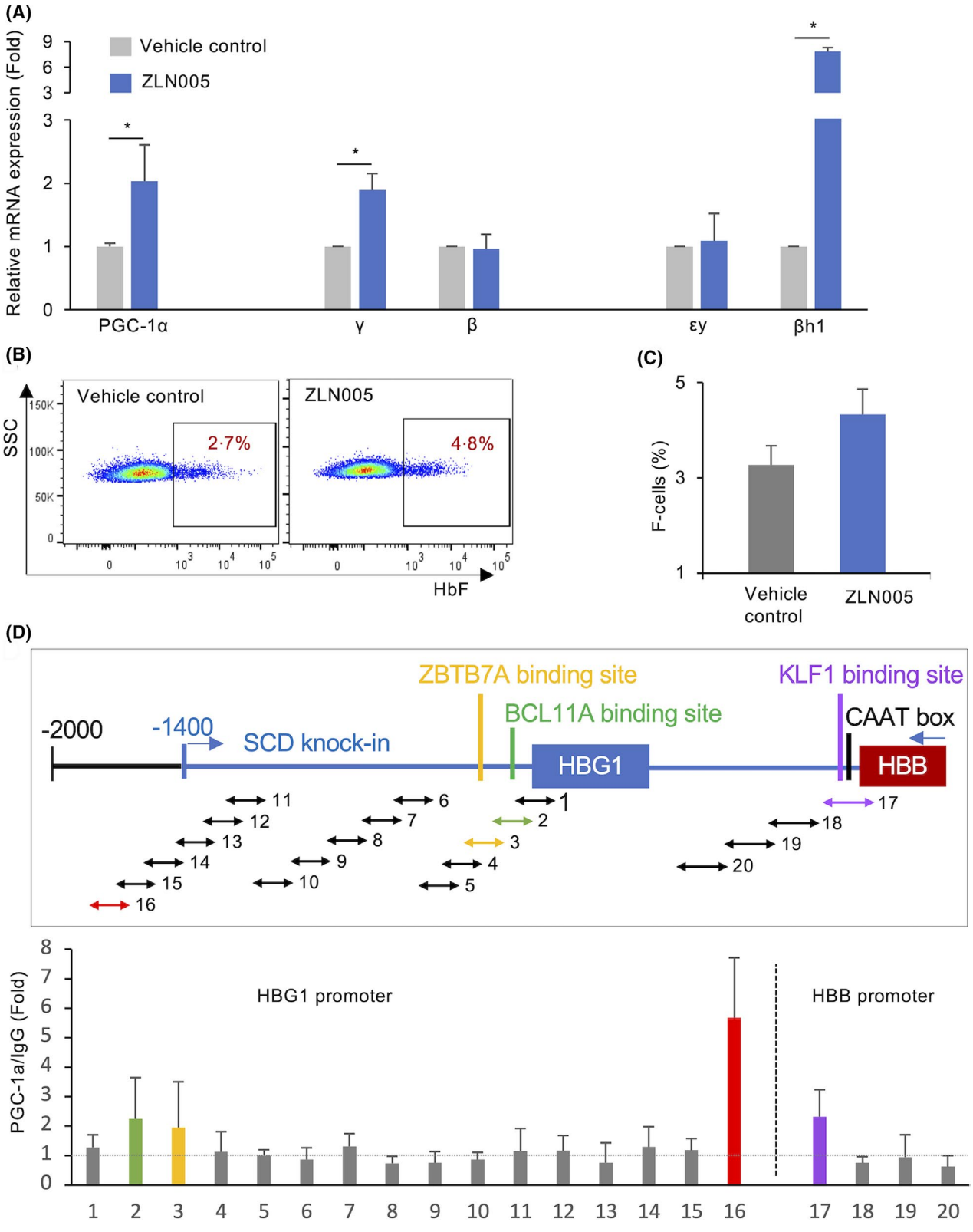


FIGURE 5 Effects of ZLN005 on sickle cell disease (SCD) mice. (A) Relative mRNA abundance of PGC-1α and globin genes in SCD mice treated with ZLN005 (1 μg/g body weight/day) or vehicle control (DMSO) for four weeks. (B) Representative flow-cytometric profiles of peripheral blood from SCD mice stained with anti-HbF antibodies. (C) Statistical analysis of the percentage of F cells by flow cytometry averaged over all samples. Data are presented as mean ± SD, $n = 3$ mice per group. (D) Schematic diagram of the β-globin locus with primers (1–20) designed to amplify discrete regions across the locus. Chromatin immunoprecipitation (ChIP) analyses of PGC-1α binding to each region normalized to control IgG are shown as a bar graph. Data are presented as mean ± SD, $n = 3$ independent experiments. The grey dot line indicates the baseline of the background [Colour figure can be viewed at wileyonlinelibrary.com]

assays using HUDEP-1 cells. PGC-1α was more strongly bound to the γ-globin promoter at position 16 (−1 765 to −1 973 bp) that is not present in the knock-in fragment in SCD mice. Notably, there were weak bindings of PGC-1α in the γ-globin promoter at positions 2 (−81 to −247 bp) and 3 (−200 to −357 bp) that contain BCL11A- or ZBTB7A-binding sites respectively.[26,27] PGC-1α was also bound weakly to the β-globin promoter at position 17 (+12 to −170 bp) that contains a CAAT box[28] and KLF1 binding site (CCACACCCT)[29] (Figure 5D).

DISCUSSION

Previously, we showed that the transcriptional co-activator PGC-1α played an important role in the maturation and survival of erythroid cells.[15] PGC-1α interacts with nuclear receptors like TR2/TR4 and binds to murine globin gene promotors.[30,31] Murine PGC-1α mutants suffer from anaemia and insufficient globin gene expression.[15] In contrast, overexpression of PGC-1α in erythrocytes obtained from humanized sickle cell mice induced γ-globin mRNA expression, which phenocopied the results of the *LSD1* inhibitor RN-1 administration.[16,32–35] These data strongly suggested that PGC-1α could be a potential molecular target in SCD patients and the modulation of PGC-1α activity might be a promising novel therapeutic approach for the treatment of SCD.

It has been reported that PGC-1α regulates gene expression in two fundamental ways. First, PGC-1α is recruited via an interaction with a DNA-binding transcription factor to the gene regulatory region. The process of docking triggers a conformational change, which allows PGC-1α to interact with histone acetyltransferase (HAT)-containing proteins, among other factors.[36] Second, the interaction between the Poll II and PGC-1α, coupled with their interaction with chromatin, results in the initiation of transcription.[36,37]

Overexpression of PGC-1α in human $CD34^+$ cells resulted in increased γ-globin mRNA expression and F cells validating its role in γ-globin induction in human adult erythroid cells (Figure 1). Interestingly, PGC-1α overexpression was associated with downregulation of the γ-globin repressors *BCL11A* and *ZBTB7A* as well as *KLF1* expression further placing PGC-1α into the human globin gene regulatory framework although the mechanisms involved are unclear and need continued study.[22,23,38]

ZLN005 is a small molecule that increases PGC-1α expression in skeletal muscle cells through the 5′-prime-adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathway.[25] Activation of this pathway can also lead to the phosphorylation of PGC-1α.[39] This post-translational modification enhances the expression of the target genes but also PGC-1α expression itself.[39,40] We now show that in erythroid cell lines, primary erythroid progenitor cells, and transgenic sickle mice, PGC-1α agonism by ZLN005 can induce γ-globin gene expression and HbF synthesis. However, it is unclear whether ZLN005 activates PGC-1α in erythroid cells through the same pathways as it does in skeletal muscle cells. ZLN005 at 1 μM but not 5 μM counteracted the effect of HU on terminal differentiation (Figure 4F), suggesting that the expression of PGC-1α might have a narrow therapeutic window for erythrocyte differentiation and haemoglobin regulation. In aggregate, our results support targeting the PGC-1α pathway as an additional means to induce HbF that could be used with HU, and that ZLN005 might serve as a lead compound for developing new HbF-inducing SCD therapeutics.

Previously, we found that the stage-specific association of PGC-1α to the βh1-globin gene promoter is closely correlated with βh1-globin gene activation. PGC-1α binds to the βh1-globin promoter at embryonic day 11.5 when βh1-globin transcripts are abundantly expressed. By embryonic day 14.5, when βh1-globin is no longer transcribed, PGC-1α binding on the βh1-globin promoter is also diminished.[15] SCD mice given ZLN005 showed slight increases of γ-globin expression and robust increases of murine endogenous embryonic βh1-globin along with upregulating of PGC-1α. This confirmed the *in vivo* inductive effects of ZLN005 on the expression of embryonic and fetal globin genes (Figure 5A). In those sickle mice, the human ^{A}γ-globin transgene was positioned to mimic its natural location and orientation between the embryonic and human adult globin genes and might be regulated as the murine β-like globin genes.[41] The ^{A}γ-globin transgene contains only 1 400 bp of 5′ flanking sequence that might not contain some regulatory elements or binding sites for PGC-1α. ChIP assays confirmed PGC-1α preferential binding 1 700–1 900 bp upstream of the ^{A}γ-globin gene transcription start site, a region not present in the transgene promoter. PGC-1α also weakly binds to the loci containing the BCL11A- or ZBTB7A-binding site on the γ-globin gene promoter, indicating possible competitive interactions among globin regulators.[26,27] Perhaps optimized ZLN005 dosing in SCD mice could increase PGC-1α expression sufficiently to overcome the HbF-repressive effects of BCL11A and ZBTB7A at the proximal promoter region. Another potential PGC-1α binding site is on the adult β-globin gene promoter CAAT box region containing the KLF1 binding site, suggesting that PGC-1α might play a role in regulating the β-globin gene that would potentially be of benefit for β-thalassaemia patients (Figure 5D).[29] This also might be the reason that β-globin mRNA expression increased upon PGC-1α overexpression in $CD34^+$ cells, although it was not statistically significant (Figure 1A).

Multiple forms of gene therapy have made good progress (up to 40%–50% HbF or anti-sickling HbA^{T87Q}). However, this modality is unlikely to soon become widely applicable due to the need for myeloablative conditioning, prolonged and intense follow-up, and off-target potential and cost, which is likely to be between $1 and $2 million for a one-time dose.[42–46] A small molecule like ZLN005, with oral bioavailability[25,47] and effects additive to HU and possibly other HbF inducers, might have more therapeutic potential where SCD is most prevalent.

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CONFLICT OF INTEREST

The authors declare they have no conflicts of interest or financial interests to disclose that are relevant to the content of this work.

AUTHOR CONTRIBUTIONS

SC supervised the work. YS, AH, CQL, NN, RC and SC designed, performed, and analysed experiments and wrote the manuscript. GM, DHKC, and MHS analysed experiments and wrote the manuscript. All authors have read, commented and approved the manuscript.

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SUPPORTING INFORMATION

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