

## Brief Report

## HEMATOPOIESIS AND STEM CELLS

Pharmacologic increase in HIF1 $\alpha$  enhances hematopoietic stem and progenitor homing and engraftmentJennifer M. Speth,<sup>1</sup> Jonathan Hoggatt,<sup>1,2</sup> Pratibha Singh,<sup>1</sup> and Louis M. Pelus<sup>1</sup><sup>1</sup>Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN; and <sup>2</sup>Department of Stem Cell and Regenerative Biology, Harvard University/Harvard Stem Cell Institute, Cambridge, MA

## Key Points

- dmPGE<sub>2</sub> stabilizes the transcription factor HIF1 $\alpha$  in stem and progenitor cells.
- Pharmacologic stabilization of HIF1 $\alpha$  increases CXCR4 and enhances stem and progenitor homing and engraftment.

Hematopoietic stem cell (HSC) transplantation is a lifesaving therapy for a number of immunologic disorders. For effective transplant, HSCs must traffic from the peripheral blood to supportive bone marrow niches. We previously showed that HSC trafficking can be enhanced by ex vivo treatment of hematopoietic grafts with 16-16 dimethyl prostaglandin E<sub>2</sub> (dmPGE<sub>2</sub>). While exploring regulatory molecules involved in dmPGE<sub>2</sub> enhancement, we found that transiently increasing the transcription factor hypoxia-inducible factor 1- $\alpha$  (HIF1 $\alpha$ ) is required for dmPGE<sub>2</sub>-enhanced CXCR4 upregulation and enhanced migration and homing of stem and progenitor cells and that pharmacologic manipulation of HIF1 $\alpha$  is also capable of enhancing homing and engraftment. We also now identify the specific hypoxia response element required for CXCR4 upregulation.

These data define a precise mechanism through which ex vivo pulse treatment with dmPGE<sub>2</sub> enhances the function of hematopoietic stem and progenitor cells; these data also define a role for hypoxia and HIF1 $\alpha$  in enhancement of hematopoietic transplantation. (*Blood*. 2014;123(2):203-207)

## Introduction

Hematopoietic stem cell (HSC) transplantation is a curative treatment of immunologic malignancies, inherited metabolic diseases, and congenital immunodeficiencies, and is an attractive method for gene therapy. Transplantation success is partly dictated by the quality and number of donor cells transplanted, and is dependent on their ability to home to bone marrow (BM) niches, self-renew and differentiate. Some sources of HSCs display reduced engraftment efficiency because of inadequate number, and/or poor homing. Identifying strategies to enhance homing and expansion of HSCs can improve transplant efficiency, particularly when HSC number is limited. It is known that prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) can stimulate hematopoietic stem and progenitor cell (HSPC) proliferation.<sup>1,2</sup> Recently, the long-acting PGE<sub>2</sub> analog 16-16 dimethyl PGE<sub>2</sub> (dmPGE<sub>2</sub>) was identified in a zebrafish chemical screen as a regulator of hematopoiesis, and ex vivo exposure to dmPGE<sub>2</sub> was shown to increase engraftment in murine and nonhuman primate models.<sup>3,4</sup> This strategy has now progressed to a phase 1 clinical trial.<sup>5</sup> We previously demonstrated that part of the mechanisms of action for PGE<sub>2</sub> were the result of increases in homing, survival, and proliferation of murine and human HSCs.<sup>6</sup> PGE<sub>2</sub> enhances HSC homing primarily by increasing CXCR4 expression on HSCs; however, the mechanism(s) whereby PGE<sub>2</sub> modulates CXCR4 and HSC homing has not been defined.

HSCs have been reported to lie in hypoxic BM niches<sup>7-10</sup> that support stabilization of hypoxia-inducible factor 1  $\alpha$  (HIF1 $\alpha$ ) within HSCs. HSCs that reside in hypoxic niches have greater hematopoietic-repopulating ability,<sup>11</sup> although recent evidence suggests that

HSCs may inherently maintain hypoxic status independently from their specific BM localization.<sup>12</sup> HIF1 $\alpha$  dose-dependently regulates HSC activity,<sup>9</sup> and intracellular oxygenation status plays a role in HSC quiescence and expansion.<sup>9,13,14</sup> Given that HIF1 $\alpha$  and hypoxia regulate CXCR4 transcription in some cancer cell lines<sup>15-20</sup> and PGE<sub>2</sub> can stabilize HIF1 $\alpha$  in prostate cancer cells,<sup>21-23</sup> we hypothesized that PGE<sub>2</sub> may increase CXCR4 and HSC engraftment through effects on HIF1 $\alpha$ . Herein, we demonstrate that PGE<sub>2</sub> stabilizes HIF1 $\alpha$  protein and transcriptional activity, which is required for enhanced HSPC homing, and identify a pharmacologic target for ex vivo enhancement of HSC function.

## Materials and methods

## Mice

Mice were bred in-house or were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained in the Indiana University School of Medicine animal facility. Conditional HIF1 $\alpha$  knockout (KO) mice were generated by breeding HIF1 $\alpha$ <sup>Flox/Flox</sup> and tamoxifen-Cre mice, then crossing hemizygous floxed pups with homozygous HIF1 $\alpha$ <sup>Flox/Flox</sup> mice. Resulting Cre<sup>+</sup> HIF1 $\alpha$ <sup>Flox/Flox</sup> mice were used. Experiments were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine. Additional materials and methods are provided in the supplemental Methods (available on the *Blood* Web site).

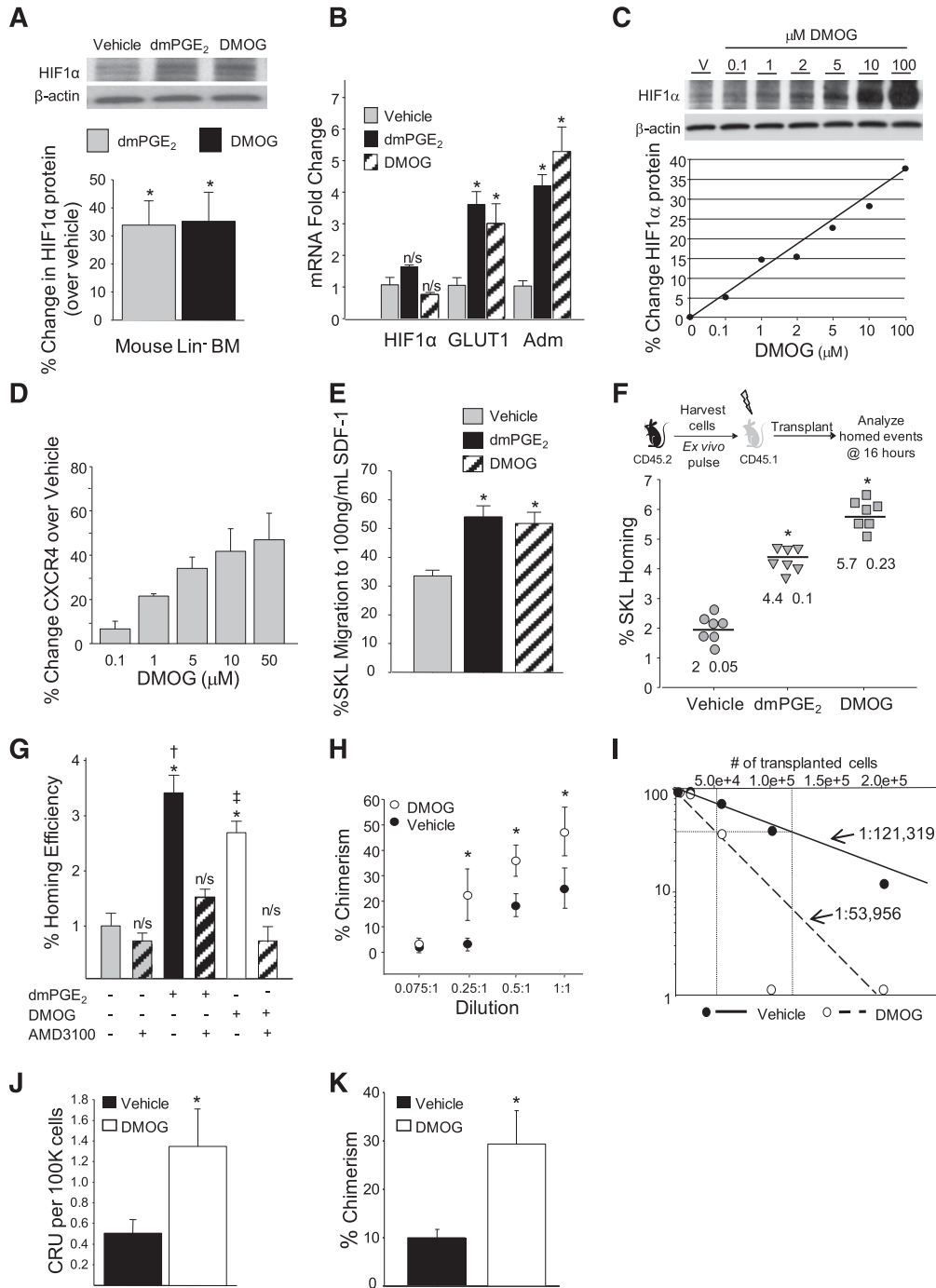
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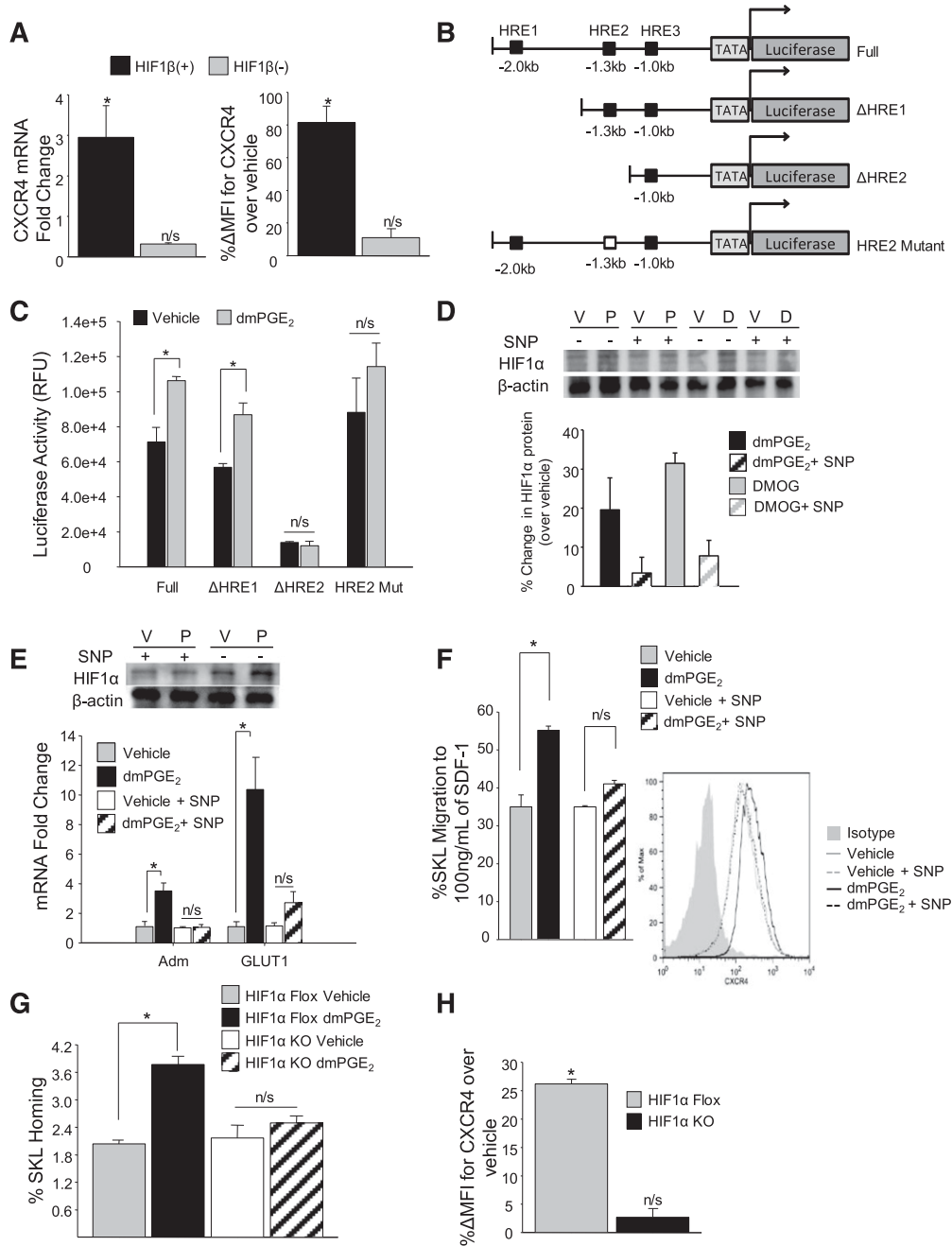
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**Figure 1. PGE<sub>2</sub> increases HIF1α protein and downstream responsive genes.** (A) (Top) Representative blot of HIF1α protein 4 hours after treatment with vehicle, 1 µM of dmPGE<sub>2</sub>, or 5 µM of DMOG. (Bottom) Densitometry analysis of HIF1α protein expression in mouse lineage<sup>neg</sup> BMCs treated with vehicle, dmPGE<sub>2</sub>, or DMOG. Data are expressed as X ± standard error of the mean (SEM) percent change in protein levels over vehicle control from 3 separate experiments. (B) Expression of HIF1 responsive genes in mouse lineage<sup>neg</sup> BMCs after treatment with dmPGE<sub>2</sub> and DMOG as determined by quantitative reverse-transcription polymerase chain reaction. Data are X ± SEM, N = 3 experiments. \*P < .05. (C) (Top) Dose-response blot of HIF1α protein 4 hours after treatment with vehicle or DMOG. (Bottom) Densitometry analysis of HIF1α protein expression in mouse lineage<sup>neg</sup> BMC. (D) Dose response of CXCR4 on SKL cells treated with vehicle or DMOG. Lineage<sup>neg</sup> BMCs were treated with DMOG for 2 hours at 37°C, washed, and incubated in RPMI with 10% heat-inactivated fetal calf serum for 16 hours. CXCR4 expression on LSK-gated cells was analyzed by flow cytometry. (E) In vitro transwell migration of murine SKL cells. One million lineage<sup>neg</sup> BMCs were treated with vehicle, dmPGE<sub>2</sub>, or DMOG. Cells were assayed for the ability to migrate to 100 ng/mL recombinant murine SDF-1 for 4 hours at 37°C. Data are expressed as X ± SEM, N = 9. (F) BMCs from CD45.1 mice were treated with vehicle, 1 µM of dmPGE<sub>2</sub>, or 5 µM of DMOG and 1 × 10<sup>6</sup> treated lineage<sup>neg</sup> cells transplanted into lethally irradiated CD45.2 mice. At 16 hours later, BM was analyzed for homed SKL cells. Data are represented as X ± SEM from 2 separate experiments (N = 4-5 mice/group/experiment, each assayed individually). (G) Similar homing experiment with or without the addition of AMD3100 10 minutes before transplantation. Data are represented as X ± SEM from 2 separate experiments (N = 4-5 mice/group/experiment, each assayed individually) \*P < .05. (H) Percent contribution (chimerism) and (I) frequency analysis for DMOG determined by Poisson statistics using LCALLC software. Vehicle P<sub>0</sub> = 121,319 and DMOG P<sub>0</sub> = 53,956. (J) Competitive repopulating units of DMOG and vehicle-treated cells in peripheral blood 6 months after transplant. Data are represented as X ± SEM from 2 pooled experiments (N = 5 mice/group/experiment, each assayed individually). (K) Secondary transplant percent chimerism at 6 months after transplant (N = 6 mice/group each assayed individually). \*P < .05.



**Figure 2. HIF1 $\alpha$  transcriptional activity is necessary for PGE<sub>2</sub>-induced CXCR4 upregulation.** (A) (Left) CXCR4 cell surface expression ( $X \pm$  SEM;  $N = 3$  experiments) on HIF1 $\beta$  (-) and HIF1 $\beta$  (+) cells 24 hours after treatment with dmPGE<sub>2</sub>. CXCR4 cell surface expression was determined by flow cytometry. Data are expressed as percent change in mean fluorescence intensity of CXCR4 over vehicle. \* $P < .05$ . (Right) CXCR4 cell surface expression ( $X \pm$  SEM;  $N = 3$  experiments) in HIF1 $\beta$  (-) and HIF1 $\beta$  (+) cells 2 hours after treatment with vehicle or dmPGE<sub>2</sub> determined by quantitative reverse-transcription polymerase chain reaction. (B) Schematic of pGL2b luciferase reporter constructs containing various regions of the murine CXCR4 promoter. (C) In vitro Luciferase reporter assay. 293T cells were transfected with either full-length CXCR4 promoter constructs containing all HREs (Full), truncated constructs containing 1 HRE ( $\Delta$ HRE1) or 2 HREs ( $\Delta$ HRE2), or a mutated 1.3 kb of HRE (HRE 2 Mut). After 24 hours, cells were split equally and treated with either vehicle or dmPGE<sub>2</sub> for 16 hours at 37°C. Luciferase activity was measured using the Firefly Luciferase assay kit (Promega). Data are represented as  $X \pm$  SEM from 2 separate experiments ( $N = 6$ ). \* $P < .05$ . (D) (Top) Representative blot of HIF1 $\alpha$  protein 4 hours after treatment with vehicle, dmPGE<sub>2</sub>, or DMOG, with or without the addition of SNP. (Bottom) Densitometry analysis of HIF1 $\alpha$  protein expression in mouse lineage<sup>neg</sup> BMCs treated with vehicle, dmPGE<sub>2</sub>, or DMOG, with or without the addition of SNP. Data are expressed as  $X \pm$  standard deviation percent change in protein levels over vehicle control from 2 separate experiments. (E) Expression of HIF1 responsive genes after treatment with vehicle or dmPGE<sub>2</sub> with or without SNP. Data are expressed as  $X \pm$  SEM,  $N = 3$  experiments. (F) (Left) In vitro transwell migration of murine SKL cells to 100 ng/mL of SDF-1. Lineage<sup>neg</sup> cells were treated with vehicle or 1  $\mu$ M of dmPGE<sub>2</sub> with or without 100  $\mu$ M of SNP for 2 hours at 37°C. Data are expressed as mean  $\pm$  SEM,  $N = 3$  experiments. \* $P < .05$ . (Right) Representative fluorescence-activated cell sorter histogram showing CXCR4 expression on SKL cells compared with isotype control. (G) In vivo homing of HIF1 $\alpha$  KO cells. BMCs from conditional HIF1 $\alpha$  KO or floxed control (CD45.2) mice were treated with vehicle or dmPGE<sub>2</sub>, and  $1 \times 10^6$  treated lineage<sup>neg</sup> cells were transplanted into lethally irradiated BoyJ (CD45.1) mice. At 16 hours later, BM was analyzed for homed SKL cells. Data are represented as  $X \pm$  SEM from 1 experiment ( $N = 4$ -5 mice/group/experiment, each assayed individually). (H) CXCR4 expression ( $X \pm$  SEM) on HIF1 $\alpha$  KO and HIF1 $\alpha$  Floxed SKL cells after treatment with dmPGE<sub>2</sub>.  $N = 3$  mice/group, each assayed individually. \* $P < .05$ .

## Results and discussion

We previously demonstrated that short-term exposure to PGE<sub>2</sub> upregulates HSPC CXCR4 and enhances their migration to stromal cell-derived factor 1 (SDF-1) and BM homing *in vivo*. However, the mechanisms whereby PGE<sub>2</sub> modulates HSPC CXCR4 and homing are unknown. In prostate cancer cells and renal tubular cells,<sup>21,22</sup> PGE<sub>2</sub> stabilizes HIF1 $\alpha$  protein without affecting messenger RNA (mRNA), and inhibiting PGE<sub>2</sub> biosynthesis reduces HIF1 $\alpha$  and HIF-responsive genes.<sup>23</sup> In HEK cells and in microglial cells, HIF1 $\alpha$  upregulates CXCR4 by interacting with hypoxia response elements (HREs) within the CXCR4 promoter.<sup>18,19</sup> We hypothesized that the enhancing effect of PGE<sub>2</sub> on HSPC CXCR4, migration, and homing could result from HIF1 $\alpha$  stabilization and enhanced HIF1 $\alpha$  transcriptional activity and that pharmacologic manipulation of HIF1 $\alpha$  may be an adjunct or alternative strategy to enhance HSC engraftment. We first determined whether PGE<sub>2</sub> stabilizes HIF1 $\alpha$  in primary BM cells (BMCs), compared with the prolyl hydroxylase domain inhibitor dimethylxylglycine (DMOG), a known HIF1 $\alpha$  stabilizer.<sup>24</sup> Pulse treatment of lineage<sup>neg</sup> BMCs with 1  $\mu$ M of dmPGE<sub>2</sub> or 5  $\mu$ M of DMOG significantly increased HIF1 $\alpha$  protein expression by ~35% (Figure 1A), with no effect on HIF1 $\alpha$  mRNA (Figure 1B). Concomitantly, mRNA levels of 2 downstream HIF1 responsive genes adrenomedullin and glucose transporter-1 were significantly increased after PGE<sub>2</sub> and DMOG treatment (Figure 1B), confirming that HIF1 $\alpha$  stabilization led to increased HIF1 $\alpha$  transcriptional activity.

Given the similar effects of dmPGE<sub>2</sub> and DMOG on HIF1 $\alpha$ , we investigated if DMOG similarly affects HSPC function. Pulse exposure of lineage<sup>neg</sup> BMC dose-dependently increased HIF1 $\alpha$  protein expression (Figure 1C) with a concomitant dose-response increase in CXCR4 levels on Sca-1<sup>+</sup> c-kit<sup>+</sup> lineage<sup>neg</sup> (SKL) cells (Figure 1D). Functionally, DMOG pulse exposure also enhanced SKL migration to SDF-1 comparable to dmPGE<sub>2</sub> (Figure 1E). In a congenic *in vivo* homing model, treatment of BMCs with DMOG enhanced SKL cell homing equivalent to dmPGE<sub>2</sub> (Figure 1F). As we reported for PGE<sub>2</sub>,<sup>6</sup> enhanced homing of SKL cells by DMOG was completely blocked by the selective CXCR4 antagonist AMD3100 (Figure 1G). In a limiting dilution, head-to-head congenic transplant model, DMOG treatment of BMC before transplantation significantly increased peripheral blood chimerism at 6 months after transplantation compared with vehicle-treated cells (Figure 1H), with no differences in lineage reconstitution between recipient, vehicle, and DMOG-treated cells (data not shown). Enhanced chimerism as a result of DMOG treatment stemmed from a twofold increase in HSC frequency (Figure 1I) and competitive repopulating units (Figure 1J). Enhanced chimerism was maintained in secondary transplants (Figure 1K). That the enhancing effects of dmPGE<sub>2</sub> on HSPC homing and engraftment can be duplicated by DMOG suggests that PGE<sub>2</sub> may mediate its effect on HSPC CXCR4 through stabilization of HIF1 $\alpha$ .

Under normoxic conditions, HIF1 $\alpha$  is ubiquitinated and targeted for proteasomal degradation.<sup>25-27</sup> In the absence of oxygen, HIF1 $\alpha$  is stabilized and translocated to the nucleus by HIF1 $\beta$ , where the translocated HIF complex interacts with HREs within the promoter of responsive genes.<sup>28</sup> To further define a HIF1 $\alpha$  requirement for PGE<sub>2</sub>-induced CXCR4 upregulation, we used mutant mouse hepatoma cells lacking or reconstituted with the HIF1 $\beta$  nuclear translocator.<sup>29</sup> In mutant hepatoma cells stably transfected with HIF1 $\beta$ ,

dmPGE<sub>2</sub> increased CXCR4 mRNA approximately threefold, and CXCR4 surface expression approximately twofold, whereas dmPGE<sub>2</sub> failed to increase CXCR4 in HIF1 $\beta$  (-) mutant cells (Figure 2A). The CXCR4 promoter contains 3 potential HREs, with the HRE located -1.3 kb from the transcriptional start site being necessary for hypoxia-induced CXCR4 upregulation in HEK cells.<sup>18</sup> Using a series of luciferase CXCR4 promoter vectors containing specific combinations of HREs (Figure 2B), a significant increase in luciferase activity was observed in dmPGE<sub>2</sub>-treated cells transfected with the vector containing the -1.3 kb of HRE. However, when the -1.3 kb of HRE was either absent ( $\Delta$ HRE2) or mutated (HRE2 Mut), no change in luciferase activity (Figure 2C) was observed after dmPGE<sub>2</sub> treatment, indicating that the -1.3 kb of HRE is required for CXCR4 regulation by both hypoxia and PGE<sub>2</sub>. Taken together, these data indicate that HIF1 $\alpha$  transcriptional activity is required for PGE<sub>2</sub>-induced gene transcription of CXCR4 and results from HIF1 $\alpha$  nuclear translocation and binding to the -1.3 kb of HRE within the CXCR4 promoter, although we cannot rule out other regulatory elements between HRE1 and HRE2 that may be contributing to CXCR4 regulation in response to hypoxia.

To support the hypothesis that HIF1 $\alpha$  is required for transcriptional upregulation of CXCR4 by PGE<sub>2</sub>, we used pharmacologic and genetic approaches to determine whether the effects of PGE<sub>2</sub> on increasing CXCR4 expression and migration to SDF-1 in primary HSPCs were mediated by HIF1 $\alpha$  stabilization. Using sodium nitroprusside (SNP), a compound that inhibits HIF1 $\alpha$  protein accumulation,<sup>30,31</sup> we saw an inhibition in both DMOG and dmPGE<sub>2</sub>-mediated HIF1 $\alpha$  stabilization (Figure 2D), as well as dmPGE<sub>2</sub>-mediated upregulation of HIF-responsive genes (Figure 2E). SNP also blocked the increase in migration to SDF-1 and CXCR4 expression, normally seen with PGE<sub>2</sub> (Figure 2F). Conditional deletion of HIF1 $\alpha$  resulted in loss of PGE<sub>2</sub>-induced enhanced HSPC homing in HIF1 $\alpha$  KO cells (Figure 2G), and no increase in CXCR4 expression after dmPGE<sub>2</sub> treatment was observed (Figure 2H), further supporting the observation that HIF1 $\alpha$  is required for PGE<sub>2</sub>-induced CXCR4 upregulation and enhanced homing.

In summary, we provide new mechanistic insight into the effects of PGE<sub>2</sub> on HSC homing and engraftment and define a new *ex vivo* pharmacologic strategy to enhance hematopoietic transplantation. This finding that HIF1 $\alpha$  stabilization by DMOG improves HSC homing and engraftment, along with recent evidence that human CD34<sup>+</sup> cells cultured in hypoxic conditions exhibit elevated levels of CXCR4<sup>32</sup> and that *in vivo* administration of DMOG enhances HSC recovery after total body irradiation,<sup>33</sup> suggests several potential therapeutic strategies targeting HIF1 $\alpha$  to improve HSC engraftment and repopulation.

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## Authorship

Contribution: J.M.S. designed and performed experiments, performed data analysis and interpretation, and wrote the manuscript;

J.H. designed research and wrote the manuscript; P.S. performed experiments; and L.M.P. designed experiments, analyzed data, and wrote the manuscript.

Conflict-of-interest disclosure: J.H. and L.M.P. have received consulting fees from Fate Therapeutics. The remaining authors declare no competing financial interests.

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