Prostaglandin E₂ enhances hematopoietic stem cell homing, survival, and proliferation

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Hematopoietic stem cell (HSC) transplantation with bone marrow, mobilized peripheral blood, or umbilical cord blood (UCB) is a proven therapy for malignant and nonmalignant hematologic diseases and metabolic disorders. Repopulation of hematopoiesis is a multistep process that can be adversely affected by the inability of HSCs to migrate/home to appropriate marrow niches or poor engrafting efficiency and self-renewal. Insight into the intrinsic and extrinsic mechanisms regulating these critical functions can lead to new strategies to improve HSC transplantation efficacy.

Prostaglandin E₂ (PGE₂) is the most abundant eicosanoid and a mediator of numerous physiological systems.¹ We and others have demonstrated regulatory roles for PGE₂ in hematopoiesis. PGE₂ dose-dependently inhibits growth of human and colony-forming units granulocytemacrophage (CFU-GM) in vitro² and myelopoiesis in vivo³ but stimulates erythroid and multilineage progenitor cells.⁴ Short-term ex vivo treatment of marrow cells with PGE₂ increases the proportion of mouse colony-forming units spleen (CFU-S)⁵ and human CFU-GM² in cell cycle. In addition, PGE₂ can stimulate production of cycling human CFU-GM from a population of quiescent cells, possibly stem cells, which is critically dependent on timing, duration of exposure, and concentration.⁹ Recently, it was shown that pulse exposure to PGE₂ ex vivo increased HSC frequency of murine bone marrow cells and enhanced kidney marrow recovery in zebrafish.¹⁰ However, although it is clear that PGE₂ can affect hematopoietic stem and progenitor cells, the mechanisms of action of PGE₂ on stem cell function have yet to be determined.

In this report, we show that PGE₂ acts directly on murine HSCs to enhance their frequency after transplantation and also provides a competitive advantage that is maintained during serial transplantation, with full multilineage reconstitution. Enhanced HSC engraftment induced by PGE₂ results from increased homing of HSCs, mediated through up-regulation of the chemokine receptor CXCR4, implicated in HSC homing,¹¹ and selective stimulation of primitive HSC survival and self-renewal associated with up-regulation of the inhibitor of apoptosis protein Survivin, required for HSC maintenance and entry into cell cycle.¹²¹³ Our studies describe novel mechanisms for enhancement of HSC function by PGE₂ and support a translational strategy to facilitate HSC transplantation.

Methods

Mice and human cord blood

C57Bl/6 (CD45.2) mice were purchased from Jackson Laboratories (Bar Harbor, ME). B6.SJL-Prkdcscid Il2rgtm1Wjl/SzJ (NS2) mice were bred in-house. Mice used in transplant studies received doxycycline feed for 30 days after transplantation. The Animal Care and Use Committee of IUSM approved all protocols. Human umbilical cord blood (UCB) was obtained from Wishard Hospital (Indianapolis, IN) with IUSM IRB approval.

Flow cytometry

All antibodies were purchased from BD Biosciences (BD, San Jose, CA) unless noted. For detection and sorting of KL and SKL cells, streptavidin conjugated with PE-Cy7 (to stain for biotinylated magnetic-activated cell separation [MACS] lineage antibodies; Miltenyi Biotec, Auburn, CA), c-kit-APC, Sca-1-PE, APC-Cy7, CD45.1-PE, CD45.2-FITC, and CD34-PE were used. For SLAM SKL, we used Sca-1-PE-Cy7, c-kit-FITC, CD150-APC (eBiosciences, San Diego, CA), CD48-biotin (eBiosciences), and streptavidin-PE. UCB CD34⁺...
cells were detected using anti-human-CD34-APC. Formultilineanalysis, APC-Cy7-Mac-1, PE-Cy7-B-220, and APC-CD3 were used. EP receptors were detected with rabbit anti-EP(1-4) antibodies (Cayman Chemicals, Ann Arbor, MI) and FITC-goat-anti-rabbit IgG (Southern Biotech, Birmingham, AL). CXCR4 expression was analyzed using streptavidin-PECy7, c-kit-APC, Sca-1-APC-Cy7, and CXCR4-PE. Apoptosis was measured with FITC–annexin-V or FITC-anti-active caspase-3. For Survivin and active caspase-3 detection, cells were permeabilized and fixed using the CytoFix/CyoPerm kit (BD) and stained with anti–active-caspase-3–FITC Flow Kit (BD) or Survivin-PE (R&D Systems, Minneapolis, MN). For cell-cycle analysis, cells were stained with Hoechst-33342 (Molecular Probes, Eugene, OR) and Pyronin-Y (Sigma-Aldrich, St Louis, MO) or FITC–BrdU Flow Kit (BD). Analyses were performed on an LSRII and sorting was performed on either a FACSAria or FACSVantage sorter (BD).

dmPGE2 pulse-exposure

16,16-Dimethyl prostaglandin E2 (dmPGE2) in methyl acetate (Cayman Chemicals) was evaporated on ice under N2, reconstituted in 100% ETOH at a final concentration of 0.1 M, and stored at −80°C. For pulse exposure, cells were incubated with dmPGE2 diluted in media, on ice, for 2 hours, with gentle vortexing every 30 minutes. After incubation, cells were washed twice in media before use. Vehicle-treated cells were processed in an identical manner, using the equivalent ETOH concentration.

Limiting dilution competitive and noncompetitive transplantation

Whole bone marrow (WBM) cells (CD45.2) were treated on ice for 2 hours with 1 μM dmPGE2 (Cayman Chemicals) or 1 × 10⁻³ % ETOH per 1 × 10⁶ cells in PBS. After incubation, cells were washed twice and mixed with 2 × 10⁴ congenic CD45.1 competitor marrow cells at various ratios and transplanted intravenously into lethally irradiated CD45.1 mice (1100-cGy split dose). Peripheral blood (PB) CD45.1 and CD45.2 cells were determined monthly by flow cytometry. For head-to-head competitive analysis, WBM from CD45.1 and CD45.2 mice was treated with vehicle or dmPGE2 and mixed with 2 × 10⁶ competitor marrow cells from CD45.1/CD45.2 mice at various ratios and transplanted into lethally irradiated CD45.1/CD45.2 mice. The proportion of CD45.1, CD45.2, and CD45.1/CD45.2 cells in PB was determined monthly. HSC frequency was quantitated by Poisson statistics using L-CALC software (StemCell Technologies, Vancouver, BC) with less than 5% contribution to chimera considered negative. Competitive repopulating units (CRUs) were calculated as described. For secondary transplantations, 2 × 10⁶ WBM from CD45.1/CD45.2 mice that had previously undergone transplantation at a 1:1 ratio at 20 weeks after transplantation was injected into lethally irradiated CD45.1/CD45.2 mice in noncompetitive fashion.

Analysis of hematopoietic stem and progenitor cell homing

WBM from CD45.2 mice was labeled with 5- (and -6-)carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes), washed, and treated with either 1 μM dmPGE2 or vehicle and 2 × 10⁶ cells were transplanted into lethally irradiated CD45.2 mice. After 16 hours, femurs and tibias were flushed, Lin− cells depleted using MACS microbeads, and Lin− cells stained for SKL and the total number of CFSE+ WBM, KL, and SKL cells was determined. For congenic homing studies, Lin− cells were treated with 1 μM dmPGE2, vehicle, or PBS and 2 × 10⁶ cells transplanted into lethally irradiated CD45.2 mice. After 16 hours, CD45.1 SKL cells in recipient BM were quantitated. For competitive homing studies, Lin− cells from CD45.2 and CD45.1 mice were fluorescent-activated cell sorting (FACS) sorted and treated with dmPGE2 or vehicle, and 3 × 10⁶ CD45.1 (vehicle or dmPGE2 treated) plus 3 × 10⁶ CD45.2 (dmPGE2 or vehicle treated) SKL cells were transplanted into lethally irradiated CD45.1/CD45.2 mice. To evaluate the role of CXCR4 in homing, Lin− CD45.2 cells were treated with vehicle or 1 μM dmPGE2 plus 10 μM AMD3100 (AnorMed, Vancouver, BC), 2 × 10⁶ treated cells injected into lethally irradiated CD45.1 mice, and homed SKL cells analyzed 16 hours after transplantation. Homing of human UCB cells was evaluated in NS2 mice. UCB mononuclear cells were isolated on Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ) and treated with either dmPGE2 or vehicle, or PBS and 2 × 10⁶ cells were transplanted into each of 5 sublethally irradiated (250 cGy) mice. Homed CD34+ cells were analyzed 16 hours after transplantation.

Expression of EP receptors, CXCR4, and Survivin

Lin−/CD45.2 marrow cells were stained for SKL, SLAM, or CD34, and each of the 4 EP receptors, and surface receptor expression on KL, SKL, SLAM SKL, and CD44+/CD45.2 SKL cells was determined by FACS. For human EP receptors, UCB CD34+ cells were positively selected with MACS microbeads and stained for CD45.2 and CD38 and each of the EP receptors, and surface receptor expression was determined by FACS. To evaluate CXCR4, Survivin, and active caspase-3, Lin− cells or CD34+ UCB was treated on ice with either 1 μM dmPGE2 or vehicle control for 2 hours, washed, and then cultured in RPMI-1640/10% HI-FBS at 37°C for 24 hours, stained for SKL (murine cells) and CXCR4, Survivin, and/or active caspase-3 as described earlier, and analyzed by FACS.

Migration assays

Chemotaxis to SDF-1 was determined using a 2-channel Costar Transwell (6.5-mm diameter, 5-μm pore; Cambridge, MA) as previously described. Briefly, dmPGE2- and vehicle-treated Lin− marrow cells were cultured in RPMI/10% HI-FBS overnight to allow for up-regulation of CXCR4, washed, resuspended at 2 × 10⁶ cells/mL in RPMI/0.5% BSA (0.1 mL was added to the top chamber of the transwells, with or without rmSDF-1α [R&D Systems] in the bottom and/or top chamber), and incubated for 4 hours at 37°C. Cells completely migrating to the bottom chamber were enumerated by flow cytometry. Percentage migration was calculated by dividing total cells migrated to the lower well by the cell input multiplied by 100. SKL cell migration was determined by comparison of the proportion of SKL cells in input and migrated populations. For UCB migration, CD34+ cells were MACS selected as described and migration assays performed as described for mouse, using rhSDF-1α (R&D Systems).

Cell-cycle analysis

For in vitro cell-cycle analysis, Lin− cells were treated with either 1 μM dmPGE2 or vehicle and cultured in Stem Cell Pro Media (StemCell Technologies) with rmSCF (50 ng/mL) (R&D Systems), rhFlt-3, and rhTPO (100 ng/mL each) (Immunex, Seattle, WA). After 20 hours, cells were stained for SLAM SKL and annexin-V or active caspase-3, and the proportion of SLAM SKL cells was determined by FACS. For human EP receptors, 4 EP receptors, and surface receptor expression on KL, SKL, SLAM SKL, and CD34neg SKL cells was determined by FACS. For human EP receptors, UCB CD34+ cells were positively selected with MACS microbeads and stained for CD45.2 and CD38 and each of the EP receptors, and surface receptor expression was determined by FACS. To evaluate CXCR4, Survivin, and active caspase-3, Lin− cells or CD34+ UCB was treated on ice with either 1 μM dmPGE2 or vehicle control for 2 hours, washed, and then cultured in RPMI-1640/10% HI-FBS at 37°C for 24 hours, stained for SKL (murine cells) and CXCR4, Survivin, and/or active caspase-3 as described earlier, and analyzed by FACS.

Quantitative-RT-PCR

Total RNA was obtained using the absolute RNA purification kit (Stratagene, La Jolla, CA). A constant amount of RNA was reverse transcribed with random primers (Promega, Madison, WI) and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega) as described. DNAse- and RNase-free water (Ambion, Austin, TX) was added to obtain a final concentration equivalent of 10 ng RNA/μL and 5 μL used for quantitative reverse-transcription–polymerase chain reaction (QRT-PCR). Primers for SYBR Green QRT-PCR were designed.
A Vehicle dmPGE₂ Competitors
CD45.1 CD45.2 CD45.1/2
Transplant into irradiated recipient

B

% Chimerism
0 10 20 30 40 50

Competitor Ratio
0.075:1 0.25:1 1:1 2.5:1

CRU per 100K Cells
0 1 2 3 4 5 7

# of transplanted cells
1:85,560 1:24,613 1:23,911

C

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<th>Weeks after transplant</th>
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<td>1:16,619</td>
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<tr>
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</table>

D

% of Total WBC

Control Vehicle dmPGE
T-Lymphoid
B-Lymphoid
Myeloid

% Chimerism

20 Weeks Primary 32 Weeks Primary 12 Weeks Secondary 24 Weeks Secondary

Figure 1.
to produce an amplicon size of 75 to 150 bp. Sequences of primers are listed in Table S1 (available on the Blood website; see the Supplemental Materials link at the top of the online article). QRT-PCR was performed using Platinum SYBR Green qPCR supermix UDG with ROX (Invitrogen, Carlsbad, CA) in an ABI-7000 (Applied Biosystems, Carlsbad, CA) or MxPro-3000 (Agilent, LaJolla, CA). Dissociation curves were determined on each analysis to confirm that only one product was obtained.

Statistical analysis

All pooled values are expressed as mean plus or minus SEM. Statistical differences were determined using the paired or unpaired 2-tailed t test function in Microsoft Excel (Redmond, WA) as appropriate.

Results

PGE2 increases LTR-HSC frequency and engraftment

We previously showed that PGE2 stimulates proliferation, cycling, and differentiation of quiescent bone marrow cells into colony-forming cells, suggesting that PGE2 enhances HSC function. However, hematopoietic repopulation in myeloablated hosts is the only true measure of HSC function. A recent report by North et al showed that pulse exposure to 16,16-dimethyl PGE2 (dmPGE2) enhanced HSC frequency when transplanted into irradiated mice.

Figure 1. PGE2 enhances hematopoietic stem cell engraftment. (A) Test bone marrow from CD45.1 or CD45.2 mice was treated with vehicle or dmPGE2, respectively. CD45.1/CD45.2 hybrid marrow cells were used as competitors. Limiting dilutions were transplanted into lethally irradiated (1100 cGy, split dose) CD45.1/CD45.2 hybrid mice and chimerism in PB was analyzed for 20 weeks. A representative flow plot detecting each cell population is shown. (B) Frequency analysis (top) for vehicle (red)– or dmPGE2 (blue)–pulsed cells, determined by Poisson statistics, at 12 weeks; PO/SEM 85 560 (vehicle) and PO/SEM 23 911 (dmPGE2 treated). Chimerism in PB and CRU analysis is shown at 12 weeks (mean/SEM). Data represent 2 pooled experiments; n 5 mice/group/experiment, each assayed individually. (*P < .05 compared with vehicle control.) (C) HSC frequency analysis in recipients of vehicle- or dmPGE2-treated bone marrow over 20 weeks. Fold change indicates increase in frequency of engraftment of dmPGE2-pulsed cells compared with vehicle. (D) Representative FACS plots of multilineage reconstitution (M indicates myeloid; B, B lymphoid; and T, T lymphoid). Multilineage analysis for primary transplantation (32 weeks) and a cohort of 4 mice that received transplants from mice that underwent primary transplantation at 20 weeks, with analysis 12 weeks later. For mice that underwent primary transplantation at 32 weeks, vehicle-treated cells were (mean  SEM) 14.1% plus or minus 3.5% M, 70.8% plus or minus 1.1% B, and 17.8% plus or minus 1.4% T, versus dmPGE2-treated cells, which were 17.5% plus or minus 2.5% M, 76.9% plus or minus 3.4% B, and 7.5% plus or minus 1.2% T. For mice that underwent secondary transplantation at 12 weeks, vehicle-treated cells were 15.7% plus or minus 5.3% M, 60.3% plus or minus 4.8% B, and 22.1% plus or minus 3.6% T, versus dmPGE2-treated cells, which were 37.0% plus or minus 6.5% M, 52.3% plus or minus 5.4% B, and 9.0% plus or minus 1.4% T. (*P < .05 vs vehicle control.) Increased chimerism of dmPGE2-treated cells versus vehicle is shown for primary transplantation at 20 weeks (time of secondary transplantation) and in a subcohort at 32 weeks (time of 12-week analysis of secondary transplantation). For secondary transplantation at 12 weeks and 24 weeks. Data for 20-week primary transplantation were from 2 pooled experiments; n 5 mice/group, each assayed individually. Data for secondary transplantations were from n 5 mice/group, each assayed individually.

Figure 2. PGE2 receptors are expressed on murine and human HSPCs. (A) Representative FACS gating of Lin− murine bone marrow showing c-kit− and Sca-1− gates and SLAM (CD150−, CD48−) and CD34 gating of SKL cells. EP1-EP4 surface receptor expression on murine KL, SKL, SLAM SKL, and CD34− SKL cells is shown. (B) Representative FACS gating of human CD34− and CD34+ UCB cells. EP surface receptor expression on CD34− and CD34+ cells is shown.
mately 4-fold increase in HSC frequency was maintained, indicating that the effect of dmPGE2 pulse-exposure was stable (Figure 1C). At 32 weeks after transplantation, reconstitution was seen for peripheral B- and T-lymphoid and myeloid lineages, with no discernible differences in lineage contribution between untreated competitor cells, dmPGE2-treated cells, or vehicle-treated cells (Figure 1D).

Marrow was harvested from primary transplant recipient animals at 20 weeks after transplantation and transplanted into secondary recipients (Figure 1D) to validate expansion and self-renewal of LTRC previously exposed to dmPGE2 and vehicle. Analysis of PB 12 and 24 weeks after secondary transplantation showed multilineage reconstitution by cells from all mice that underwent transplantation, clearly demonstrating the self-renewal of primary transplanted LTRC. Unlike the primary transplantation, multilineage reconstitution by dmPGE2-treated cells showed an elevated myeloid lineage reconstitution. The increase in chimerism resulting from dmPGE2 exposure seen in primary donors was also observed in secondary transplants without any additional treatments.

Murine and human hematopoietic stem and progenitor cells express PGE2 receptors

PGE2 interacts with 4 specific, highly conserved G-protein-coupled receptors: EP1-EP4.17 Although EP receptor expression has been observed in dendritic cells,18 monocytes,19 and early zebrafish hematopoietic tissue,20 EP receptor expression on hematopoietic stem and progenitor cell populations is not known. Analysis of EP receptors on c-kit+ Linneg (KL) cells, enriched for hematopoietic progenitor cells; Sca-1+ c-kit+ Linneg (SKL) cells, enriched for multipotent progenitor cells as well as HSCs; and SLAM (CD150+, CD48+) SKL and CD34+ SKL cells, highly enriched for primitive repopulating HSCs21-22 showed that all 4 EP receptors are expressed on these hematopoietic cell populations (Figure 2A). Analogous to murine cells, all 4 receptors are expressed on human CD34+ UCB cells enriched for HSCs and CD34+, CD38- cells that contain the most primitive human HSCs (Figure 2B). Quantitative RT-PCR showed that mRNA for all 4 EP receptors is detected in the whole bone marrow cell population and in FAC-sorted KL, SKL, and primitive CD34+ SKL cells (Figure 3A) and on common lymphoid progenitor cells (CLPs) (Linneg+, c-kitlow, Sca-1low, IL7R+) and myeloid progenitor cells (CMPs) (Linneg+, c-kit+, Sca-1-, CD34+) (not shown). Similarly, QRT-PCR analysis detected mRNA for all 4 EP receptors in purified CD34+ and CD34+, CD38- UCB cells (Figure 3B).

PGE2 increases HSC homing efficiency

Enhanced HSC engraftment by PGE2 could result from increased HSC number and/or cell-cycle status,23 effects on facilitating cells,24 or effects on HSC homing or proliferation in the host...
Figure 4. PGE₂ ENHANCES HSC FUNCTION

A. Label and ex vivo treat CD45.2
   Transplant CD45.2
   Analyze homed events @ 16 hours
   NS
   % CFSE +
   Vehicle
   dmPGE₂
   WBM KL SKL
   P < .02

B. Ex vivo treatment CD45.1
   Transplant CD45.2
   Analyze homed events @ 16 hours
   NS
   % Homing Efficiency SKL cells
   Fold Change
   Vehicle
   dmPGE₂
   P < .05
   P < .001

C. Vehicle dmPGE₂
   CD45.1
   Transplant CD45.1/2
   CD45.2
   CD45.1
   dmPGE₂ Vehicle
   CD45.1
   Transplant CD45.1/2
   CD45.2

D. % Homing Efficiency CD45.1
   CD34+ UCB cells
   Vehicle
   dmPGE₂
   P < .05
   P < .001

Figure 4.
Figure 5.

### A

- **Homing Efficiency SKL Cells**
  - Vehicle
  - dmPGE$_2$

### B

- **% ∆MFI for CXCR4**
  - KL
  - SKL

### C

- **% ∆MFI for CXCR4**
  - UCB CD34$	ext{+}$

### D

- **% Migration to 100ng/mL SDF-1**
  - Vehicle
  - dmPGE$_2$

**Figure 5.**
PGE₂ ENHANCES HSC FUNCTION 5451

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PGE₂ increases hematopoietic stem and progenitor cell CXCR4 and chemotaxis to SDF-1α

The stromal cell–derived factor-1α (SDF-1α)/CXCR4 axis is believed to play a major role in hematopoietic stem and progenitor cell (HSPC) trafficking and chemotaxis/homing to the bone marrow microenvironment. In addition, up-regulation of CXCR4 on human CD34⁺ cells and endothelial cells by PGE₂ has been reported, and PGE₂ can increase monocyte chemotaxis to SDF-1α. We therefore evaluated whether the mechanism of improved homing of dmPGE₂-treated HSPCs resulted from up-regulation of SDF-1α/CXCR4 signaling. Pulse exposure to dmPGE₂ increased CXCR4 expression on KL and SKL cells (Figure 5A). Similarly, dmPGE₂ increased CXCR4 expression on CD34⁺ UCB cells. QRT-PCR demonstrated elevated CXCR4 mRNA levels in dmPGE₂-treated SKL cells compared with vehicle (2.65-fold).

In vitro, HSPCs selectively migrate to a gradient of SDF-1α, a process that is believed to reflect their marrow-homing capacity. We evaluated the effect of dmPGE₂ treatment on HSC chemotaxis to a gradient of SDF-1 in an vitro transwell migration assay to determine whether PGE₂-mediated CXCR4 up-regulation enhanced chemotaxis. Both vehicle- and dmPGE₂-treated SKL cells demonstrated significant migration to 1 to 1000 ng/mL SDF-1, however, chemotaxis was significantly higher in cells treated with dmPGE₂ (Figure 5B). Analysis of positive and negative gradients indicated that the dmPGE₂-enhancing effect on SKL cell chemotaxis did not result from a nonspecific increase in chemokinesis (Figure 5B top inset). Enhanced migration to SDF-1 by dmPGE₂ was also observed using FACS-sorted SKL cells, suggesting a direct effect on HSCs (Figure 5B bottom inset). Chemotaxis of UCB CD34⁺ cells to SDF-1 was also significantly enhanced by pulse exposure to dmPGE₂ and migration was blocked by the selective CXCR4 antagonist AMD3100, indicating a specific effect mediated through the CXCR4 receptor (Figure 5C).

To specifically determine whether up-regulated CXCR4 played a role in the enhanced homing observed after dmPGE₂ treatment, cells were treated with AMD3100 prior to evaluation of in vivo homing. PGE₂ pulse-exposure increased homing of SKL cells as described earlier, and incubation of vehicle or dmPGE₂-pulsed cells with AMD3100 significantly reduced SKL cell homing (Figure 5D) and abrogated the improved homing efficiency of dmPGE₂-pulsed cells. Pulse exposure to dmPGE₂ enhanced SKL cell homing efficiency by 2.6- plus or minus 0.3-fold (P < 0.05), which was reduced to 1.3- plus or minus 0.2-fold (P = NS) in the presence of AMD3100. AMD3100 reduced overall homing by 42% plus or minus 5% (range, 31%-64%), consistent with previous reports.

PGE₂ decreases HSC apoptosis and increases Survivin

PGE₂ treatment produces an approximately 4-fold increase in HSC and CRU frequency (Figure 1), but only an approximately 2-fold enhancement in homing (Figure 4), which suggests that other mechanisms are involved in enhanced engraftment. Apoptosis is an important regulatory process in normal and malignant hematopoiesis, and PGE₂ has been implicated in antiapoptotic signaling. Moreover, activation of cAMP, a downstream signaling molecule of EP receptors, inhibits apoptosis in CD34⁺ cells. We hypothesized that PGE₂ treatment affects survival and/or proliferation of HSCs, contributing to enhanced engraftment. Under conditions of reduced serum concentration, dmPGE₂ pulse-exposure significantly reduced intracellular active caspase-3 in SLAM SKL cells.
Figure 6. PGE2 decreases apoptosis, increases Survivin expression, and decreases active caspase-3 in SKL cells. (A) Lin<sup>-</sup> bone marrow cells were treated with dmPGE<sub>2</sub> or vehicle and cultured in media supplemented with 2% FBS without growth factors for 24 hours to induce apoptosis. Cultured cells were stained for SKL and SLAM and PE-anti–active caspase-3 and/or FITC–annexin-V and the proportion of SKL or SLAM cells undergoing apoptosis was determined by FACS. (Inset) dose-response analysis of the effects of dmPGE<sub>2</sub> on SKL cell apoptosis. (B) Fold increase in intracellular Survivin levels (mean fluorescence intensity [MFI]) in control and dmPGE<sub>2</sub>-pulsed murine SKL and human CD34<sup>+</sup> UCB cells (1.7- and 2.4-fold, respectively) compared with control (Figure 6B). QRT-PCR analysis of treated SKL cells similarly indicated elevated Survivin mRNA compared with control (2.94-fold). In a kinetic analysis, decreased active caspase-3 coincident with an increase in Survivin was seen at 24, 48, and 72 hours after exposure of SKL cells to dmPGE<sub>2</sub> compared with control (Figure 6C), consistent with the caspase-3 inhibiting activity of Survivin.

**PGE2 increases HSC proliferation**

In previous reports, we showed that Survivin regulates HSC entry into and progression through cell cycle. Furthermore, β-catenin, implicated in HSC proliferation and self-renewal, lies downstream of EP receptor pathways. The ability of PGE<sub>2</sub> to modulate these cell-cycle regulators suggests that an increase in HSC self-renewal and proliferation might contribute to the enhanced engraftment of dmPGE<sub>2</sub>-pulsed cells. To test this hypothesis, we analyzed the cell-cycle status of dmPGE<sub>2</sub>- or vehicle-pulsed SKL cells in vitro. Pulse exposure to dmPGE<sub>2</sub> increased SKL cell cycling (Figure 7A), with 60% more SKL cells in G<sub>1</sub> + S/G<sub>2</sub>M phase of the cell cycle after dmPGE<sub>2</sub> treatment compared with controls. To evaluate the effect of PGE2 exposure on primitive, quiescent HSCs, we performed additional in vitro studies using SLAM SKL cells. Similar to SKL cells, in vitro dmPGE<sub>2</sub> pulse-exposure significantly increased the proportion of SLAM SKL cells in cell cycle (G<sub>1</sub> + S/G<sub>2</sub>M) by 24% (Table 1). No significant effect on cell-cycle rate of KL or Lin<sup>-</sup> cells was seen (not shown), suggesting that dmPGE<sub>2</sub> selectively increases HSC cycling state.

To confirm the effect of dmPGE<sub>2</sub> on enhancement of HSC cell cycle observed in vitro, bone marrow cells were pulsed with dmPGE<sub>2</sub> and injected into congenic mice treated with BrdU after transplantation, and the proportion of donor BrdU<sup>+</sup> SKL cells was determined 16 hours later (Figure 7B). An approximately 2-fold increase in the proportion of homed SKL cells in S + G<sub>2</sub>/M phase was observed for cells pulsed with dmPGE<sub>2</sub> prior to transplantation, confirming that short-term exposure of HSCs to dmPGE<sub>2</sub> stimulates HSCs to enter and progress through cell cycle in vivo.

**Discussion**

It is well documented that PGE<sub>2</sub> participates in regulation of hematopoiesis, inhibiting myelopoiesis both in vitro and in vivo; promoting erythroid and multilineage colony formation; and enhancing proliferation of CFU-S<sup>7</sup> and CFU-GM<sup>8</sup>. In addition, PGE<sub>2</sub> stimulates production of cycling hematopoietic progenitor cells (HPCs) from the quiescent bone marrow compartment<sup>9</sup>, suggesting that PGE<sub>2</sub> has biphasic effects on hematopoiesis. These studies implicated PGE<sub>2</sub> in stem cell function, but did not directly evaluate HSCs. Moreover, one cannot rule out that inhibition of colony formation by PGE<sub>2</sub> resulted from modulation of HSC commitment to self-renewal versus differentiation, thus reducing colony formation. Recently, ex vivo exposure of bone marrow cells to PGE<sub>2</sub> was shown to facilitate murine hematopoietic cell engraftment<sup>10</sup>, validating previous studies that PGE<sub>2</sub> enhances
HPC production and extending the role of PGE₂ to stimulation of HSC function. However, the mechanism by which PGE₂ produced this effect was not defined. We now demonstrate, for the first time, that PGE₂ has direct and stable effects on long-term repopulating HSCs, as determined by serial transplantation, and facilitates HSC engraftment by increasing CXCR4, enhancing migration to SDF-1 and homing to bone marrow, up-regulating Survivin expression that blocks HSC apoptosis, and increasing the proportion of LTR-HSCs entering into and progressing through cell cycle.

Direct comparison in competitive transplant models showed that short-term exposure of HSCs to PGE₂ produces an approximately 4-fold competitive advantage, consistent with published results. However, previous studies showed a maximal effect on HSC frequency at 12 weeks after transplantation with reduced HSC frequency at 24 weeks, suggesting a greater effect on short-term rather than long-term repopulating cells. Our studies show that PGE₂-induced enhancement of HSC frequency was stable throughout a more than 20-week period and was maintained in secondary transplantations through 24 weeks, clearly indicating a sustained effect on LTRC. The reasons for this difference in repopulating stability are not clear, but may relate to more precise head-to-head quantitation of HSC competition in our model.

Full hematopoietic reconstitution was observed in recipients of serial transplantations using either control- or PGE₂-treated cells, indicating no adverse impact of PGE₂ on HSC self-renewal. In fact, a trend toward increased LTRC activity was seen, indicating that the enhancing effect of short-term PGE₂ exposure on HSCs observed in primary transplantations was long-lasting, since no additional treatment was performed on cells or animals before secondary transplantations. Although it is commonly assumed that a single HSC compartment gives rise to all hematopoietic lineages, recent studies have demonstrated the presence of normal HSCs.

Table 1. Effects of short-term in vitro exposure of SLAM SKL cells to dmPGE₂ on cell cycle

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<th>In vitro treatment</th>
<th>G₀</th>
<th>G₁</th>
<th>S + G₂M</th>
<th>% cells in cycle†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>63.4 ± 2.5</td>
<td>2.6 ± 0.7</td>
<td>33.8 ± 2.1</td>
<td>36.4 ± 2.4</td>
</tr>
<tr>
<td>1 μM dmPGE₂</td>
<td>54.8 ± 2.2†</td>
<td>6.8 ± 1.9†</td>
<td>38.4 ± 1.6†</td>
<td>45.2 ± 2.2‡</td>
</tr>
</tbody>
</table>

*Lin⁻ cells, treated with either 1 μM dmPGE₂ or vehicle for 2 hours and cultured in the presence of growth factors (50 ng/mL rmSCF, 100 ng/mL each of rhFlt-3 and rhTPO) for 20 hours, were stained with SLAM SKL, Hoechst-33342, and Pyronin-Y and the proportion of SLAM SKL cells in G₀, G₁, S, and G₂M phase of the cell cycle was determined by quantitation of DNA and RNA content by FACS. Data are mean plus or minus SEM for n = 9 mice, each assayed individually.

†Percentage of cells in G₀ + S + G₂M; combined data for n = 9 mice.

‡P < .05 compared with vehicle control.
biased toward lymphoid or myeloid differentiation. In secondary transplantsations, we observed a myeloid bias in mice that received a transplant of PGE2-treated HSCs, suggesting a possible selective effect of PGE2 on myeloid-biased HSCs. However, white blood cell counts in mice that underwent secondary transplantation have remained within normal ranges. Continued analysis of mice that have undergone transplantation and retransplantation studies will validate these findings and determine their significance, if any.

Although it was suggested that PGE2 does not affect HSC homing, earlier studies evaluated WBM and did not specifically assess HSPCs. When evaluating total transplanted cells, we also observed no difference in homing efficiency between control- and PGE2-treated cells; however, enhanced homing of SKL cells by PGE2 was clearly evident. Furthermore, enhanced homing efficiency of PGE2-treated, sorted SKL cells was observed, suggesting a direct effect on HSCs. PGE2 also enhanced homing of human CD34+ UCB in immunodeficient NS2 mice, strongly indicating translation of HSC enhancement to human stem cell products. Although more primitive populations of HSCs than defined by SKL can be identified (eg, CD34+ SKL and SLAM SKL), the small number of homed events that can ultimately be detected using these markers precludes the ability to define effects of PGE2 on these extremely rare cells in vivo in individual mice as we performed. The fact that we see similar activities of PGE2 on LTRC and on SKL and SLAM SKL cells in several assays of HSC function without significant effects on the progenitor cell–enriched KL cell population indicates that the SKL cell fraction is a valid indicator of the effects of PGE2 on HSC homing.

PGE2 treatment increased SKL CXCR4 mRNA and surface expression, consistent with effects of PGE2 on CXCR4 in CD34+ cells. This increase in CXCR4 corresponds directly with a functional increase in chemotaxis to SDF-1, and chemotaxis was blocked using AMD3100. In addition, AMD3100 significantly reduced the enhancing effect of PGE2 on homing in vivo, suggesting that increased CXCR4 expression and chemotraffaction to narrow SDF-1 are largely responsible for the enhanced homing effect, although additional effects on adhesion molecule expression or function cannot be excluded. We also found elevated mRNA and protein levels of Survivin, with concomitant reduced active caspase-3 in PGE2-treated SLAM SKL cells. It is likely that enhanced HSC survival, mediated through Survivin, also contributes to enhanced engraftment.

Pulse exposure to PGE2 increased the proportion of HSCs in cell cycle by approximately 2-fold, with increased frequency of HSCs, CRUs, and homed BrdU+ SKL cells and maintenance of enhanced HSC frequency in primary and secondary transplantations, suggesting that PGE2 pulse-exposure initiated at least a single round of HSC self-renewal. EP2 and EP4 receptor activation can result in phosphorylation of glycogen synthase kinase-3 (GSK-3) and increased β-catenin signaling, which is downstream of the Wnt pathway that has been implicated in HSPC survival and self-renewal. Signaling by PGE2, through EP4 can directly increase β-catenin, and synergistic cross-talk between COX-2 and Wnt pathways has been suggested. Further exploration of specific signaling pathways and EP receptors involved in mediating the effects of PGE2 may refine our understanding of the role of PGE2 on HSC function. Although it has been suggested that cycling cells have reduced narrow homing, which may be the result of triggered apoptosis, it is clear that PGE2-treated cells have both enhanced homing and enhanced migration, despite their enhanced cycling. This may be explained by the increase in CXCR4 migratory response overcoming deficits in the homing of cycling cells and/or increased homing occurring before an increase in cycling. In addition, PGE2 may protect homed cycling HSCs from apoptosis, thus allowing for simultaneous enhanced homing, survival, and proliferation in these cells.

We previously reported that Survivin is required for HSCs to enter and progress through cell cycle, and deletion in conditional knockout mice indicates Survivin is required for HSC maintenance. Survivin also facilitates HSPC cell cycle through p21WAF1/CIP1, known to be involved in HSC function, and blocks caspase-3 activity, recently implicated in HSC self-renewal. Our findings that PGE2 up-regulates Survivin, which is consistent with previous reports in cancer and dendritic cells, and decreases intracellular levels of active caspase-3 in primitive HSCs suggest that the Survivin pathway may also be involved in the effects of PGE2 on self-renewal. It is interesting to note that transcription of both Survivin and CXCR4 is up-regulated by the transcription factor hypoxia-inducible factor-1α (HIF-1α), which can be stabilized by PGE2, potentially linking these PGE2 responsive pathways.

In summary, we have defined specific mechanisms of action and new pathways for enhancement and regulation of HSC function by PGE2. The 4-fold increase in HSC frequency and engraftment produced by exposure to PGE2 results from the cumulative effect of a 2-fold increase in HSC homing and a 2-fold increase in HSC cell-cycle activity. Although the precise signaling pathways remain to be determined, enhanced engraftment involves up-regulation of CXCR4 and Survivin, with subsequent increased chemotactic response to SDF-1 and reduced apoptosis. The ability to easily improve homing and survival/proliferation of HSCs by short-term PGE2 exposure is exciting from a clinical perspective, especially in transplantation settings where insufficient or low HSC numbers are found (eg, UCB and some mobilized peripheral blood stem cell products). Our limiting dilution transplant studies show that equivalent engraftment is achieved with one fourth the number of PGE2-treated cells compared with controls, supporting a use for PGE2 when HSC numbers are limiting. Homing and migration studies using UCB CD34+ cells clearly suggest potential translation to human hematopoietic grafts. Lastly, it will be interesting to determine whether enhanced engraftment/recovery can be achieved by administering PGE2 in vivo or if PGE2 used in vivo can further facilitate engraftment of HSCs exposed to PGE2 ex vivo. In COX2 knockout mice, recovery from 5-fluorouracil (5-FU) is delayed, suggesting that COX2 activation and subsequent PGE2 production may be critical for HSC expansion.

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Authorship
Contribution: J.H. designed and performed research, collected and analyzed data, performed statistical analysis, and wrote the paper; P.S. designed and performed research and collected and analyzed data; J.S. participated in performance of research and collection and analysis of data; L.M.P. participated in designing research,
References


Prostaglandin E$_2$ enhances hematopoietic stem cell homing, survival, and proliferation

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