Prostaglandin E2 Enhances Aged Hematopoietic Stem Cell Function

Andrea M. Patterson (anmapatt@iupui.edu)
Indiana University School of Medicine https://orcid.org/0000-0002-3000-1157
P. Artur Plett
Indiana University School of Medicine
Carol H. Sampson
Indiana University School of Medicine
Edward Simpson
Indiana University School of Medicine
Yunlong Liu
Indiana University School of Medicine
Louis M. Pelus
Indiana University School of Medicine
Christie M. Orschell
Indiana University School of Medicine

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Abstract

Aging of hematopoiesis is associated with increased frequency and clonality of hematopoietic stem cells (HSCs), along with functional compromise and myeloid bias, with donor age being a significant variable in survival after HSC transplantation. No clinical methods currently exist to enhance aged HSC function, and little is known regarding how aging affects molecular responses of HSCs to biological stimuli. Exposure of HSCs from young fish, mice, nonhuman primates, and humans to 16,16-dimethyl prostaglandin E_2 ($dmPGE_2$) enhances transplantation, but the effect of $dmPGE_2$ on aged HSCs is unknown. Here we show that ex vivo pulse of bone marrow cells from young adult (3 mo) and aged (25 mo) mice with $dmPGE_2$ prior to serial competitive transplantation significantly enhanced long-term repopulation from aged grafts in primary and secondary transplantation (27% increase in chimerism) to a similar degree as young grafts (21% increase in chimerism; both $p<0.05$). RNA sequencing of phenotypically-isolated HSCs indicated that the molecular responses to $dmPGE_2$ are similar in young and old, including CREB1 activation and increased cell survival and homeostasis. Common genes within these pathways identified likely key mediators of HSC enhancement by $dmPGE_2$ and age-related signaling differences. HSC expression of the PGE_2 receptor EP4, implicated in HSC function, increased with age in both mRNA and surface protein. This work suggests that aging does not alter the major $dmPGE_2$ response pathways in HSCs which mediate enhancement of both young and old HSC function, with significant implications for expanding the therapeutic potential of elderly HSC transplantation.

Introduction

Hematopoietic stem cells (HSCs) are responsible for continual replacement of all blood cell types, and HSC transplantation (HSCT) is a life-saving option for many patients with hematologic diseases. However, HSC function and engraftment capacity drastically decrease with age[1–7]. Loss of immune function, anemia, myeloid skewing, and increased incidence of myeloproliferative diseases and leukemia are observed in the elderly, and aged bone marrow (BM) cells are unfavorable for transplantation[4,8–11]. While many factors are thought to contribute to HSC aging, including cell-intrinsic and niche-mediated[2,12,13], specific molecular pathways functionally linked to aged HSC defects are not well characterized, and no treatment exists to enhance aging hematopoiesis or augment the transplantation potential of these cells. The number of people > 65 years of age is projected to almost double between 2012 and 2050[14], intensifying the problem of aged HSC dysfunction and the critical need for novel therapeutic approaches for those in need of HSC support.

Prostaglandin E2 (PGE_2) is a bioactive lipid with hematopoietic roles described since the 1970s[15–17]. More recently, the stable derivative 16,16-dimethyl PGE_2 ($dmPGE_2$) was found to enhance HSC frequency and transplantation efficiency in both zebrafish and murine models[18,19]. We have previously shown that pulse exposure to $dmPGE_2$ enhances homing, survival, and proliferation for both young (2–3 mo) mouse BM and human cord blood derived CD34 + cells, which was associated with increased CXCR4 and

Survivin expression, decreased apoptosis, and increased HSC proliferation[19,20]. Further, $dmPGE_2$ pulse exposure augmented competitiveness of human cord blood grafts in a Phase I clinical trial with double cord blood transplantation[21], and has been used clinically to enhance engraftment of gene modified HSCs[22]. Thus, $dmPGE_2$ is known to enhance HSC function and has potential for clinical translation.

The objective of the current work was to assess the effects of $dmPGE_2$ pulse exposure on aged HSCs at both the functional and molecular level in comparison to young HSCs. Functionally, $dmPGE_2$ pulse prior to competitive serial transplantation significantly enhanced the long-term repopulating capacity of aged HSCs similarly to young. A high-throughput genomic comparison of old and young HSC responses to $dmPGE_2$ highlighted common transcriptional pathways in old and young mediating HSC enhancement, and identified novel signaling alterations in HSCs with age.

Materials And Methods

Mice

All studies were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. Aged mice: C57BL/6J male mice were purchased at 10 weeks old from Jackson Laboratories (Bar Harbor, ME) and aged in our facility until used at 25 mo of age. Young mice: C57BL/6J male mice were purchased at 10 weeks old from the Indiana University In Vivo Therapeutics Core (IVTC) and used at 3 mo of age. Transplant recipient mice: B6.BoyJ congenic (CD45.1) male and female mice were purchased at 6–8 weeks of age from the IVTC and used as transplant recipients at 8–10 weeks of age. Transplants were randomized by recipient sex and age such that young and old donor samples were equally distributed between recipients that were male or female, and younger (closer to 8 weeks) or older (closer to 10 weeks). Mice were randomized within cages so that each cage contained recipients of both vehicle and $dmPGE_2$-pulsed cells, but from the same donor when possible for rigor of comparison between matched samples.

BM Collection

Femurs, tibiae, pelvic bones, and humeri from young and old C57BL/6J mice were flushed with cell buffer (PBS containing 2% FBS and 2 mM EDTA). Cells were passed through a 40 μm filter, and total nucleated cells (TNC) enumerated using an Element HT5 Hematology Analyzer (Heska Corporation, Loveland, CO). In the transplantation experiment, an aliquot of cells from each mouse was removed for flow cytometric assessment of young vs. old BM prior to $dmPGE_2$ pulse.

$DmPGE_2$ Pulse Exposure

$DmPGE_2$ in methyl acetate from Cayman Chemicals (Ann Arbor, MI) was stored at -20 ^{o}C. Prior to use, $dmPGE_2$ was evaporated to dryness on ice under N_2 and reconstituted in 100% EtOH at a stock concentration of 10 mg/ml (26.28 mM). Whole BM (WBM) cells (transplantation experiments) or lineage-

depleted WBM cells (RNA-seq experiments) from individual C57BL/6J (CD45.2) young or old mice were split in two portions. One portion was pulsed in a concentration of 10 μM $dmPGE_2$ in cell buffer, and the other in an equivalent volume of vehicle (100% EtOH) in cell buffer, at 2.5 x 10^6 TNC/mL for 1 h in a humidified CO_2 incubator at 37°C, with vortexing every 15 min. Cells were then centrifuged at 500 x g for 10 min to remove $dmPGE_2$ or vehicle and washed with cell buffer.

Competitive Serial Transplantation

Pulsed WBM cells from 4 young and 4 old donor C57BL/6J mice (CD45.2) were transplanted into 6 recipients per donor, where 3 received cells pulsed with $dmPGE_2$ and 3 received cells from the same donor pulsed with vehicle for matched analysis. To that end, 8–10 week-old congenic recipient mice (CD45.1) were exposed to ^{137}Cs irradiation (11 Gy split dose, 4 h apart) using a Mark 1 Irradiator (JL Shepherd, San Fernando, California), as previously described[23]. $DmPGE_2$- or vehicle-pulsed donor WBM cells were resuspended in PBS and combined with competitor CD45.1 WBM cells not pulsed with $dmPGE_2$ or vehicle in a 3:2 ratio, for a final retro-orbital injection of 100 uL containing 3 x 10^5 donor and 2 x 10^5 competitor TNCs per recipient mouse. Recipients were given autoclaved acidified water (pH 2.0–3.0) and irradiated Uniprim diet (Envigo, Madison, WI) for 1 week prior to, and 4 weeks after irradiation/transplantation. Peripheral blood (PB) was analyzed monthly for donor chimerism and for multilineage reconstitution at month 6.

After 7 mo, WBM was collected from all primary recipients. Cells from each set of 3 replicate primary recipients (or 2 in two cases where one recipient died in primary phase) were combined equally and 1.5 x 10^6 TNC transplanted into 3 secondary CD45.1 recipients conditioned with radiation as above. PB was analyzed monthly for donor chimerism, except for months 4–5 due to pandemic-related laboratory restrictions, and for multilineage reconstitution at month 6.

Cell Staining and Flow Cytometry Analysis

BM cells were stained with an amine-reactive live/dead dye (Invitrogen LIVE/DEAD Fixable Dead Cell Stain kit Yellow, Thermo Fisher, Waltham, MA) followed by blocking with TruStain FcX+ (Biolegend, San Diego, CA) and fluorophore-conjugated monoclonal antibodies to identify phenotypic HSCs (pHSCs) using a lineage cocktail in APC (CD3, GR-1, B220, Ter-119, CD11b, and CD5; R&D Systems, Minneapolis, MN), Sca1 in PerCP/Cy5.5, c-Kit in BV711, CD150 in PE/Cy7, and CD48 in APC/Cy7 (Biolegend). EP4 was stained with an unconjugated primary polyclonal antibody (Novus Biologicals, Littleton, CO) and a secondary anti-rabbit IgG antibody conjugated to BV421 (Biolegend). Cells were then analyzed immediately by flow cytometry.

PB cells were RBC-lysed, blocked with TruStain FcX+ (Biolegend), and stained with fluorophore-conjugated antibodies to white blood cell markers including CD45.1 in PE, CD45.2 in FITC, B220 in Pacific Blue, CD3 in PerCP/Cy5.5, and CD11b in APC (Biolegend). Cells were fixed in 1% paraformaldehyde and stored at 4°C until flow cytometric analysis. Flow cytometric data were acquired using an LSRII flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (BD Biosciences).

WBM Processing, HSC Sorting, and RNA Extraction for Sequencing

WBM cells from 8 young and 4 old mice were enriched for immature cells by magnetic lineage depletion (EasySep Mouse Hematopoietic Progenitor Cell Isolation kit, STEMCELL Technologies), then pairs of young samples were combined to increase the number of pHSCs available for sorting from 4 individual BM samples from young mice alongside 4 individual BM samples from old mice. Cells were then pulsed with dmPGE$_2$ or vehicle for 1 h as described above, immediately stained, and viable pHSCs isolated by fluorescence associated cell sorting (FACS) using a SORP Aria flow cytometer (BD Biosciences). Cells were sorted directly into lysis buffer for RNA extraction using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). A 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) was used for quality control of all RNA preparations, giving a median RNA integrity of 9.6 RIN (range 6.2–10.0).

RNA Sequencing and Statistics/Bioinformatics

Library preparation was performed using the SMART-Seq v4 Ultra Low Input RNA Kit (Clontech, Mountain View, CA) and the Nextera XT DNA Lib Kit (Illumina, San Diego, CA). Two hundred picomolar pooled libraries were utilized per flow cell for clustering amplification on cBot using HiSeq 3000/4000 PE Cluster Kit and sequenced with 2×75bp paired-end configuration on HiSeq4000 (Illumina) using a HiSeq 3000/4000 PE SBS Kit. Sequencing data were assessed for quality using FastQC version 0.11.5 (Babraham Bioinformatics, Cambridge, UK). The sequence reads were mapped to the mouse genome (UCSC mm10) using STAR (Spliced Transcripts Alignment to a Reference)[24] version 2.5 using parameter "–outSAMmapqUnique 60". To evaluate the quality of the RNA-seq data, number of reads that fall into different annotated regions (exonic, intronic, splicing junction, intergenic, promoter, UTR, etc.) of the reference genes were determined with bamUtils[25] version 0.5.9.

Uniquely mapped sequencing reads were assigned to mm10 refGene genes and quantified using featureCounts (from subread)[26] version 1.5.1 using parameters "-s 2 -p -Q 10". Low quality mapped reads (including reads mapped to multiple positions) were excluded. Differential expression (DE) analysis was performed with edgeR[27]. In this workflow, the statistical methodology applied uses negative binomial generalized linear models with likelihood ratio tests. A paired design was used to compare samples across conditions such that the relationships between samples from the same animals were retained ("blocking" in edgeR). False discovery rate (FDR) calculations therefore reflect the collective significance of the treatment on gene expression while accounting for baseline differences between the animals.

DE data was analyzed for biological insights using Ingenuity Pathway Analysis (IPA, Qiagen, Hilden, Germany)[28]. Two IPA Core Analyses were run, one for the young and one for the old dmPGE$_2$ vs. vehicle comparisons, with cutoffs set to FDR < 0.05 and $\log_2$FC>|0.5| (FC, fold change). The young and old analyses were compared using the IPA Comparison Analysis function. Upstream Regulators were filtered on Genes, RNAs, and Proteins, z-score cutoff |2|, p-value cutoff 1.3 ($\log_{10}$). Diseases and Functions were filtered on Cellular and Molecular Functions, with additional removal of cancer cell-specific functions, z-

score cutoff |2|, p-value cutoff 1.3 (log_{10}). The set of 70 genes increased by $dmPGE_2$ in young (FDR < 0.05) but not in old (FDR > 0.6) was submitted to the DAVID 6.8 Functional Annotation tool using default settings and output from the Functional Annotation Chart filtered on UP_KEYWORDS and cutoff at Benjamini-adjusted p-value < 0.01 (david.ncifcrf.gov)[29].

Heatmaps were generated in Microsoft Excel using conditional formatting for cell color based on FPKM values (fragments per kilobase per million mapped reads). Heatmaps depicting relative gene expression among all samples/groups (blue-red) used the following formula for each cell: (FPKM – average FPKM across all samples all groups)/standard deviation of FPKM across all samples all groups. Heatmaps depicting change in (Δ) gene expression between paired samples (gray-orange) used the formula: $(FPKM^{dmPGE2} – FPKM^{vehicle})$/standard deviation of FPKM across all samples in age group, where superscripts denote different treatments of the same mouse BM sample.

Statistics

Other statistical analyses were performed using Microsoft Excel and GraphPad Prism 8. All data with error bars represent mean ± SEM. Paired t-tests were performed between matched vehicle- and $dmPGE_2$-treated samples from the same donor in chimerism analyses, 1-tailed for expected increase with $dmPGE_2$. Unpaired t-tests were performed between young and old flow cytometry data points (2-tailed).

Results

$DmPGE_2$ enhances long-term serial repopulation capacity of aged HSCs

Since $dmPGE_2$ enhances homing, survival and proliferation of long-term repopulating HSCs in young mice[19,20], we tested whether a similar effect could be demonstrated on HSCs from old mice, which are known to demonstrate reduced regenerative potential and myeloid skewed differentiation[1,30]. WBM aliquots from 3 mo (young) or 25 mo (old) mice were pulsed with either $dmPGE_2$ or vehicle prior to competitive serial transplantation, allowing for matched analysis of $dmPGE_2$ effects on long-term repopulating HSC potential (Fig. 1A). PB chimerism in recipients was assessed monthly by flow cytometry for CD45.2 donor cell frequency and for trilineage distribution at 6 mo post-transplant (Fig. 1B). In primary transplants, $dmPGE_2$ pulse increased long-term donor-derived chimerism for all 4 young donors and 3 of 4 old donors (Fig. 1C). After secondary transplantation, long-term repopulation was increased by $dmPGE_2$ pulse for all donors of both age groups (Fig. 1D). Chimerism of aged grafts at 6 mo post-secondary transplant was increased an average of 27%, similar to the average increase of 21% for young (Fig. 1E), indicating enhanced HSC self-renewal capacity for both young and aged BM grafts by $dmPGE_2$.

Lineage reconstitution

Analysis of PB lineage reconstitution showed that pulse with $dmPGE_2$ increased myeloid frequency in all four young grafts, observed in both primary and secondary transplantations (Fig. 1C-D, dark green). However, this was not at the expense of lymphoid production; total lymphoid and myeloid production were both increased by $dmPGE_2$ pulse exposure in comparison to competitor cells as a frequency of total peripheral CD45 cells (CD45.1 + CD45.2) (Fig. 1F). Transplantation of grafts from old mice resulted in myeloid-skewed PB reconstitution as expected, regardless of vehicle or $dmPGE_2$ treatment (Fig. 1C-D, dark green). However, similar to young, both lymphoid and myeloid production were increased by $dmPGE_2$ in comparison to competitor cells (Fig. 1F). One old graft lost long-term myeloid production after secondary transplantation, with poor overall chimerism for both $dmPGE_2$ and vehicle (Old 4, Fig. 1D), but still showed increased lymphoid potential with $dmPGE_2$ (Fig. 1F), which resulted in an apparent reversal of myeloid skew among donor cells in these recipients.

Donor BM phenotypic HSC (pHSC) frequencies and EP4 expression pre-transplant

Prior to pulse and transplantation, aliquots of WBM cells from young and old donors were analyzed for baseline pHSC frequency and $dmPGE_2$ receptor expression (Fig. 2A). As expected, the pHSC population in old mice was greatly expanded compared to that in young mice, exhibiting 24-fold higher frequency (Fig. 2B), similar to our previously published data[23]. EP4, the receptor primarily implicated in HSC functional responses to $dmPGE_2$[31–33], was strongly expressed on the surface of aged HSCs and was higher compared to young (Fig. 2C).

BM chimerism and stem/progenitor frequencies post-transplant

Donor-derived BM chimerism was assessed at the time of secondary transplantation (7 mo post-primary transplant). CD45.2 BM chimerism was significantly higher after $dmPGE_2$ pulse for all grafts from young donors, and was variably increased after $dmPGE_2$ pulse for 3 of 4 grafts from old donors (Fig. 2D), mirroring the effect on primary PB chimerism. The single old donor without increased BM chimerism also did not show increased PB chimerism after primary transplantation (“Old 1”, Fig. 1C). However, the subsequent superiority of the $dmPGE_2$-treated graft in secondary transplantation (“Old 1”, Fig. 1D) suggests it may retain a $dmPGE_2$-mediated qualitative advantage in HSC self-renewal revealed by the stress of serial transplantation.

While the aged grafts contained 24-fold higher pHSC frequency compared to young grafts prior to pulse and transplantation (Fig. 2B), similar pHSC frequencies were found among long-term engrafted CD45.2 BM cells from young and old donors (Fig. 2E), likely reflecting the functional compromise of the original pHSC population in the aged mice. Interestingly, $dmPGE_2$ increased the frequency of phenotypic myeloid-committed progenitors (pMP) (gating illustrated in Fig. 2A) among engrafted donor cells for 4 of 4 young grafts and 3 of 4 old grafts (Fig. 2F). Further, $dmPGE_2$ altered the distribution of stem versus progenitor

cells within the primitive LSK compartment of the old grafts, partially reversing the age-associated predominance of pHSCs (Fig. 2G) in favor of increased phenotypic hematopoietic progenitor cells (pHPCs, Fig. 2H; gating illustrated in Fig. 2A). This may suggest increased capacity for generation and/or maintenance of downstream progenitor populations when grafts are pulsed with $dmPGE_2$.

Shared and divergent transcriptional responses to $dmPGE_2$ in young and old HSCs

To identify molecular correlates of enhanced long-term HSC function by $dmPGE_2$ in both young and aged HSCs, and to identify signaling differences in HSCs with age, pHSCs from pulsed BM samples were immediately isolated by FACS and analyzed using RNA-seq (Fig. 3A). Differentially expressed genes were defined as FDR < 0.05 by pair-wise analysis between $dmPGE_2$- and vehicle-pulsed samples from the same mouse, across 4 young samples or 4 old samples to determine age-specific transcriptional effects. The paired analyses allow for robust detection of $dmPGE_2$ effects despite baseline gene expression differences between individual mice, which can become particularly variable with advanced age.

$DmPGE_2$ significantly affected 230 genes in young HSCs (184 increased, 46 decreased), and 112 genes in old HSCs (85 increased, 27 decreased). Of these, 53 common genes reached significance in both age groups (49 increased, 4 decreased; Fig. S1). Substantially fewer genes reached FDR < 0.05 in old HSCs, which may be due in part to greater variability in responsiveness among old mice, but may also reflect an overall decrease in HSC responses to $dmPGE_2$ with age. All differentially expressed genes reaching FDR < 0.05 in both age groups combined were clustered based on similar (age-independent) or unique (age-dependent) $dmPGE_2$ effects between young and old, and were visualized both for relative expression across all samples (Fig. 3B) and for individually paired Δ gene expression induced by $dmPGE_2$ (Fig. 3C). The top two clusters in Figs. 3B and 3C represent similar increase/decrease in both age groups (FDR < 0.05 for at least one age group and < 0.6 in the other), and comprised a majority of the genes. Since $dmPGE_2$ pulse effectively enhanced HSC function in serial transplantation for both age groups, we first focused analysis on shared gene effects to narrow the pathways likely involved in the mechanism of enhancement. The lower clusters represent genes affected differently by $dmPGE_2$ in old and young HSCs; these are not likely involved in the mechanism of enhancement but can shed light on changes in HSC signaling pathways with age.

Shared $dmPGE_2$ signaling in young and old HSCs

IPA was used to identify the most likely upstream regulators activated by $dmPGE_2$ in HSCs based on all downstream gene expression changes (FDR < 0.05 and $\log_2$FC>|0.5|) in young and old mice, and a comparison analysis of results from each age group revealed the same top regulators predicted in young and old (Fig. 4A). The regulator with the highest activation z-score in both age groups was CREB1, a known mediator of PGE_2 signaling through receptors EP2 and EP4[34], supporting similar HSC-intrinsic

signaling in old and young. Among the CREB1-regulated genes contributing to each z-score, 16 were shared between young and old (Fig. 4B).

Cellular functions activated by dmPGE$_2$ were also predicted, with *'Cell survival'* and *'Cellular homeostasis'* reaching significant activation z-scores in both young and old HSCs (Fig. 4C). Overlapping gene sets contributed to both functions, and 18 of these genes were shared between young and old (Fig. 4D). Seven of these genes (bold) are also known to be regulated by CREB1 (Fig. 4B). Thus, CREB1 activation by dmPGE$_2$ may be increasing HSC survival and homeostasis through *Bhlhe40, Cdkn1a, Cebpb, Gadd45b, Nr4a2, Pim3*, and *Vegfa*, among others in these heatmaps potentially not yet functionally linked. Overall, these genes and predicted regulators (Fig. 4A) shared in old and young HSC responses to dmPGE$_2$ provide strong candidates for further study as molecular mediators of enhancement of HSC potential.

Since dmPGE$_2$ activates CREB1 through either EP2 or EP4, baseline mRNA expression of each receptor was compared (Fig. 4E). EP2 was not detectable above background in young or old HSCs while EP4 was highly expressed and increased with age, confirming the flow cytometry findings for EP4 (Fig. 2C). Also, dmPGE$_2$ pulse exposure decreased EP4 expression in each of the old HSC samples and 3 of 4 young samples (Fig. 4F). Desensitization through EP4 has been noted[35], and this negative feedback effect on EP4 expression has been reported in murine HSCs after in vivo dmPGE$_2$ treatment[36]. Together these findings further support EP4 as the relevant receptor for enhancement of both young and old HSC long-term function.

Divergent dmPGE$_2$ signaling in young and old HSCs

The third cluster in Fig. 3B/C, and the second largest cluster overall, is comprised of 70 genes significantly increased by dmPGE$_2$ in young HSCs (FDR < 0.05) but not affected at all in old HSCs (FDR > 0.6). Visualizing relative expression in Fig. 3B, many of these genes appear already elevated at baseline in vehicle-treated old HSCs and are not further increased with dmPGE$_2$. These genes were classified for functional annotation enrichments using DAVID bioinformatics analysis (Table 1). The top enrichment category was 'Phosphoprotein', comprising 44 of the 70 genes. These phosphoproteins also made up 23/29 genes from 'Alternative splicing', and 14/17 genes from 'Transcription', the next two most enriched categories. The category of 'Alternative Splicing' is defined by UniProt as "Protein for which at least two isoforms exist due to distinct pre-mRNA splicing events" (uniprot.org). Since alternative splicing has been implicated in the development of myeloproliferative disorders which increase in prevalence with age[37,38], these genes are reported in Table 2. Thus, a sizeable subset of dmPGE$_2$-induced genes observed in young HSCs become less responsive with age and are enriched for phosphoproteins with alternative splice variants and those involved in transcriptional regulation.

Table 1. Functional Annotation enrichments[a] among the 70 genes increased by dmPGE$_2$ in young but not in old HSCs

#	Term	Count (/70)	Fold Enrich.	Benj. p-val
1	Phosphoprotein	44	1.9	3.7E-5
2	Alternative splicing	29	2.0	4.2E-3
3	Transcription	17	3.1	4.9E-3
4	Transcription regulation	16	3.0	6.1E-3
5	Transferase	15	3.0	7.1E-3
6	Coiled coil	21	2.3	8.4E-3

[a]DAVID Functional Annotation Chart, filtered on UP_KEYWORD

and Benjamini-adjusted $p < 0.01$.

Table 2
Genes annotated with 'Alternative splicing'

#	Gene	$DmPGE_2$ Effect		Avg Baseline (Veh) FPKM[a]		
		FDR Yng	FDR Old	Yng	Old	FC with age
1	*Adgrg2*	0.0433	0.9989	1.06	6.16	5.81
2	*Evc*	0.0002	0.7657	1.23	6.14	5.00
3	*Sytl5*	0.0434	0.9956	1.09	2.82	2.58
4	*Dmxl2*	0.0244	0.9961	1.19	2.89	2.43
5	*Ttc39a*	0.0433	0.8046	1.57	3.46	2.21
6	*Ubr4*	0.0039	0.8811	6.44	10.40	1.61
7	*Clk1*	0.0475	0.8251	28.02	43.76	1.56
8	*Dmd*	0.0407	0.7699	0.73	1.12	1.53
9	*Epc1*	0.0076	0.9400	8.40	12.86	1.53
10	*Mga*	0.0407	0.9750	7.99	12.08	1.51
11	*4932438a13rik*	0.0096	0.3003	5.39	8.09	1.50
12	*Kmt2c*	0.0113	0.9757	5.67	8.43	1.49
13	*Mycbp2*	0.0080	0.1822	6.70	9.87	1.47
14	*Adgrl2*	0.0067	0.9859	11.57	17.01	1.47
15	*Usp53*	0.0286	1.0000	2.35	3.35	1.43
16	*Suco*	0.0052	0.6945	8.85	12.50	1.41
17	*Madd*	0.0235	0.8872	27.58	37.94	1.38
18	*Dopey1*	0.0363	0.7969	3.60	4.66	1.30
19	*Slc12a7*	0.0489	0.8411	12.54	15.98	1.27
20	*Ankrd6*	0.0249	0.8225	0.80	1.02	1.27
21	*Ggt5*	0.0313	1.0000	2.68	3.39	1.27
22	*Gramd1a*	0.0052	0.8862	38.00	47.70	1.26

[a]Average FPKM (fragments per kilobase per million mapped reads) among vehicle-treated young (Yng) or old HSCs (n = 4 per sex); FDR, false discovery rate for $dmPGE_2$ vs. vehicle analyses; FC, fold change.

#	Gene	$DmPGE_2$ Effect		Avg Baseline (Veh) FPKM[a]		
		FDR Yng	FDR Old	Yng	Old	FC with age
23	*Birc6*	0.0216	0.9706	5.13	6.43	1.25
24	*Syne2*	0.0016	1.0000	1.58	1.93	1.22
25	*Zfp292*	0.0328	0.9640	8.55	10.19	1.19
26	*Setdb1*	0.0153	0.7388	14.53	16.61	1.14
27	*Ipo8*	0.0112	0.7760	14.05	15.78	1.12
28	*Smcr8*	0.0282	0.9233	5.19	5.40	1.04
29	*Pcgf5*	0.0433	0.7128	47.00	42.89	0.91

[a]Average FPKM (fragments per kilobase per million mapped reads) among vehicle-treated young (Yng) or old HSCs (n = 4 per sex); FDR, false discovery rate for $dmPGE_2$ vs. vehicle analyses; FC, fold change.

An additional "difference of difference" FDR calculation was utilized to compare the treatment effect in old versus young HSCs and identify genes affected significantly differently by $dmPGE_2$ in each age group (Fig. 5A). Each of these genes was affected in the opposite direction by $dmPGE_2$ in old and young HSCs (FDR < 0.05). Several had higher average expression with age in the vehicle-treated samples and were decreased by $dmPGE_2$ in old HSCs, as opposed to being increased by $dmPGE_2$ in young HSCs, including *Ccbe1, Evc, Mlk2, Mycbp2, Rorb, and Ubr4*. Since *Rorb* was so strongly upregulated with age and differentially affected by $dmPGE_2$, its close family members *Rora* and *Rorc* were also examined (Fig. 5B). *Rora* was slightly increased with age and strongly upregulated by $dmPGE_2$ in both young and old HSC, while *Rorc* was strongly increased with age and slightly elevated by $dmPGE_2$ in old but not in young. In addition, two genes (*H2afx* and *Kcnj5*) had lower average expression with age and were increased by $dmPGE_2$ in old HSCs, opposite to the $dmPGE_2$ effect in young, and four others had similar baseline expression in young and old HSCs but opposite treatment effects (*Elf2, Max, Psap, and Ncoa2*; Fig. 5A). These RNA-seq analyses identify potential molecular targets for age-related HSC dysfunction.

Discussion

After HLA matching, donor age is generally the most critical factor determining survival after HSCT[11]. Grafts from older donors have been associated with increased graft failure even when controlling for cell number[39,40], which may relate to declining inherent stem cell quality[1–5,41]. Increased incidence of graft versus host disease (GVHD) in recipients of grafts from older donors has also been observed[10,42], potentially related to an increase in antigen-experienced lymphocytes with age[43,44] or the general increase

in low-level inflammation that characterizes aging[45]. However, neither of these associations show consistent relationships with HSCT outcome[10,46], and a combination of several age-related factors likely contribute. For these reasons, transplant physicians tend to favor younger donors, but finding the appropriately matched donor can sometimes be difficult or impossible. The probability of finding an unrelated matched donor varies with ethnicity, and can be especially problematic for some ethnic groups, e.g. African-Americans[47]. Elderly family members would have strong motivation to donate if suitably matched, and strategies to enhance aged grafts could help increase the possibility of success.

While hematopoietic malignancies treatable by autologous transplant increase in prevalence with age, elderly patients are often not eligible due to the rigors of the HSCT process as well as their own declining HSC quality. However, recent strategies employing reduced-intensity conditioning for elderly patients, as well as advances in transplant technique and supportive care, increasingly enable allogeneic and autologous HSCT in this population[48–50]. The ability to augment the function of aged grafts prior to infusion could facilitate successful, life-saving autologous transplants for older patients. Here we found that ex vivo pulse exposure to dmPGE$_2$ can enhance the transplantation capacity of murine HSCs of advanced age. Previous work in young grafts has shown this effect to translate from zebrafish and mice[18,19] to non-human primates[51,52] and ultimately to enhancement of human cord blood transplantation[21]. Thus, the current findings have a high likelihood of translation.

PGE$_2$ is an eicosanoid synthesized within most body tissues by many different cell types, acting in autocrine or paracrine fashion[53]. Activities mediated by PGE$_2$ are highly pleiotropic, depending on the tissue/cell type and expression of its four G-protein coupled receptors EP1-4[35,54]. EP1 signals primarily through PKC and Ca^{2+} mobilization, EP2 and EP4 induce cAMP production and subsequent cAMP response element-binding protein (CREB) activation as observed here, and EP3 inhibits cAMP production[35,54]. EP4 has been recognized as a key functional regulator for HSCs, including transplantation studies[32,33]. In the setting of radiation exposure, where dmPGE$_2$ protects and enhances HSC function[36], only dmPGE$_2$ or EP4 agonism conferred survival from lethal irradiation[55]. In the current transcriptomic analysis, the predominance of CREB1-induced gene expression following dmPGE$_2$ pulse in both young and old HSCs, along with strong EP4 expression but undetectable EP2 mRNA levels as observed here and previously by RNA-seq of purified murine pHSCs[36], strongly supports EP4 as the relevant receptor mediating HSC enhancement regardless of age.

An interesting finding in this study was increased EP4 expression in HSCs with age. PGE$_2$ production is known to increase with age in macrophages[56] and decrease with age in gastrointestinal tissues[57]. In skeletal muscle, the capacity for PGE$_2$ synthesis increases with age while receptor levels are downregulated[58]. It remains unclear if basal PGE$_2$ levels change with age within the BM, and which factors would drive the increase in EP4 expression on HSCs. The intensity of CREB1-regulated genes was noticeably higher in old HSCs after dmPGE$_2$ pulse and may be related to the number of EP4 receptors, though higher basal expression of these genes was also seen in control HSCs in a variable manner

between aged mice (Fig. 4B). Thus, the change in CREB1-regulated genes was greater in some old mice but not in others, and the relevance of increased EP4 expression on aged HSCs remains uncertain. Ultimately, this investigation established that HSCs of advanced age do not lose expression or signaling through the pivotal EP4 receptor.

While the primary objective of these studies was not to compare old versus young HSC function, but rather to evaluate the effect of $dmPGE_2$ on old grafts in parallel with young grafts, the transplantation experiments were performed simultaneously with the same cohorts of recipients and competitor cells. The studies indicated that the old and young grafts functioned similarly in regard to overall long-term and serial chimerism capacity. Since the old grafts contained approximately 24-fold higher pHSC frequency, and equivalent numbers of WBM cells were transplanted, these observations are in line with the reported substantial decrease in function of pHSCs with age[2,3,6,7]. We also observed myeloid-skewed reconstitution from aged HSCs as described[2–4, 6,30]. Interestingly, $dmPGE_2$ pulse consistently increased the relative myeloid contribution of the young donor cells, bringing their lineage ratios closer to those of the aged. However, $dmPGE_2$ also increased the overall frequencies of donor lymphoid cells in comparison to competitors, suggesting $dmPGE_2$ has a positive effect on both major immune cell branches but augments myeloid reconstitution to a greater degree. $DmPGE_2$ also augmented both branches for old HSCs compared to competitors without further affecting the inherent myeloid skew with age. Of interest, we have previously reported an increase in the proportion of myeloid cells in PB of mice transplanted with young HSCs pulsed with $dmPGE_2$ following primary and secondary transplant, however this was not consistent across tertiary and quaternary transplants and was without overall effect on the enhancement of induced stem cell competitiveness[59].

Bioinformatic analysis of $dmPGE_2$ signaling in young versus old HSCs revealed that the core response pathways remained largely unchanged with age. Several age-independent genes were identified as both increased by CREB1 signaling and involved in the significantly predicted functions of *'Cell survival'* and *'Cellular homeostasis'*. Many of these genes additionally have described roles in hematopoiesis, supporting their involvement in HSC modulation by $dmPGE_2$. *Vegfa* encodes for vascular endothelial growth factor A (VEGF-A) which, while first discovered for its primary role in angiogenesis[60], enhances human HPC formation[61], promotes hematopoietic cell generation from embryonic stem cells of both mouse[62] and human[63], and regulates HSC survival[64]. *Cebpb* is the gene for CCAAT enhancer binding protein beta (C/EBPβ), a transcription factor that promotes lymphopoiesis[65] and emergency myelopoiesis[66,67] at the level of stem and progenitor cell regulation[68,69]. *Gadd45b*, encoding growth arrest and DNA-damage-inducible beta (GADD45β), appears essential for DNA damage protection and survival of HSCs/HPCs and induced pluripotent stem cells (iPSCs) under stress[70]. *Cdkn1a* encodes the cyclin-dependent kinase inhibitor P21, which preserves HSC quiescence under stress and promotes HSC self-renewal in serial transplantation[71], while *Nr4a2* encoding the transcription factor nuclear receptor subfamily 4 group A member 2, also known as NURR1, also attenuates HSC cycling[72] and may contribute to the maintenance of stem cell quiescence during the stress of transplantation.

In addition to transplantation, steady-state hematopoiesis in older humans is subject to increased bone marrow failure and decreased hematologic tolerance of cytotoxic injury, as well as the increased propensity for myeloproliferative disorders and cancerous transformation[8,9]. A broader understanding of aged HSC function is essential to development of novel treatments for hematopoietic compromise in the elderly. The high-throughput genomic comparison of old and young HSC responses to $dmPGE_2$ provided a unique modality for investigating changes in HSC stimulation response pathways with age. Several signaling alterations identified here may have relevance for targeting in treatment of age-induced HSC defects.

Genes differentially affected by $dmPGE_2$ in young and old HSCs included numerous phosphoproteins induced in young but not in old, many of which were already elevated with age. IPA did not return any significant predictions for a common upstream regulator controlling these genes (no more than 5 had shared association with any given regulator), but functional categories including 'Alternative splicing' exhibited significant enrichment (Table 1). Abnormalities in alternative splicing have been implicated in the development of myeloproliferative disorders that increase in prevalence with age, with over 50% of myelodysplastic syndromes harboring spliceosome factor mutations in the dominant clone[37,38]. The current analysis suggests $dmPGE_2$-responsive genes that become less responsive with age tend to be those with alternative splice variants, and tend to have higher mRNA levels detectable in old HSCs pre-stimulation (Table 2). However, the current experimental design did not distinguish between splice variants, and further investigation is needed to determine whether these transcripts could be affected by dysregulated splicing in HSCs of advanced age.

Several specific genes were identified as significantly *oppositely* affected by $dmPGE_2$ stimulation in old versus young HSCs, revealing divergent molecular responses potentially related to aging defects. *Ccbe1* and *Rorb* were particularly elevated with age and strongly decreased by $dmPGE_2$ only in old HSCs. *Ccbe1*, encoding for collagen and calcium binding EGF domains 1 (CCBE1), is a secreted protein thought to function in remodeling of extracellular matrix and cell migration, and is an important factor in lymphangiogenesis[73–75]. It is also an essential mediator of erythroblastic island formation for erythropoiesis in fetal liver, though it is not required for postnatal erythropoiesis[76]. This gene has otherwise not been associated with hematopoiesis, and gene expression levels found here in young HSCs were near-zero at baseline with a very slight elevation by $dmPGE_2$. However, the *Ccbe1* transcript was much more detectable in aged HSCs and was strongly and consistently downregulated by $dmPGE_2$ stimulation. Thus, transcription of Ccbe1 appears to be 'turned on' in HSCs by an unknown aging factor that may be sensitive to 'turning back off' by $dmPGE_2$ signaling. However, a potential role for this protein in aged HSCs remains to be explored.

A more substantial target may be *Rorb*, which encodes for RAR related orphan receptor B (RORβ), a member of the highly conserved ROR family of receptor tyrosine kinases[77]. These kinases, including RORβ, are known to negatively regulate WNT/B-catenin signaling[78,79], an important facilitator of HSC fate decisions[80]. In the context of $dmPGE_2$ stimulation, $dmPGE_2$ enhanced WNT signaling during zebrafish

embryogenesis and was required for WNT-mediated regulation of HSC development[81]. In addition, RORβ is elevated with age in marrow-derived osteoprogenitor cells, contributing to development of osteoporosis[79,82]. Our study reveals that RORβ is elevated with age in HSCs, and decreased in response to $dmPGE_2$ in an age-dependent manner. *Rora* and *Rorc* also exhibited unique expression patterns affected by both age and $dmPGE_2$ treatment (Fig. 5B). Together, these findings may indicate a novel mechanism of age-associated dysregulation of HSC fate decisions through increased ROR expression, and reveal an intriguing avenue for downregulation of RORβ in aged HSCs through the PGE_2 signaling pathway.

In conclusion, this study has identified that aged HSCs primarily retain the molecular capacity to respond to $dmPGE_2$ pulse exposure and initiate transcriptional programs enhancing survival and long-term repopulating function, which has potential importance toward the goal of enhancing aged human grafts for transplantation. Moreover, age-related alterations in HSC signaling in response to PGE_2 were identified as potential targets for treatment of age-related defects.

Declarations

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Author contributions: All authors made substantial contributions to this study. AMP conceived, designed, and performed studies, acquired and analyzed data, interpreted results, visualized the data and prepared the figures, and wrote the manuscript. PAP and CHS contributed to study design and methodology, performed studies, and acquired data. ES and YL performed RNA sequencing and analysis. LMP and CMO contributed to study conception and design and critically revised the manuscript. All authors read and approved the final manuscript.

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Figures

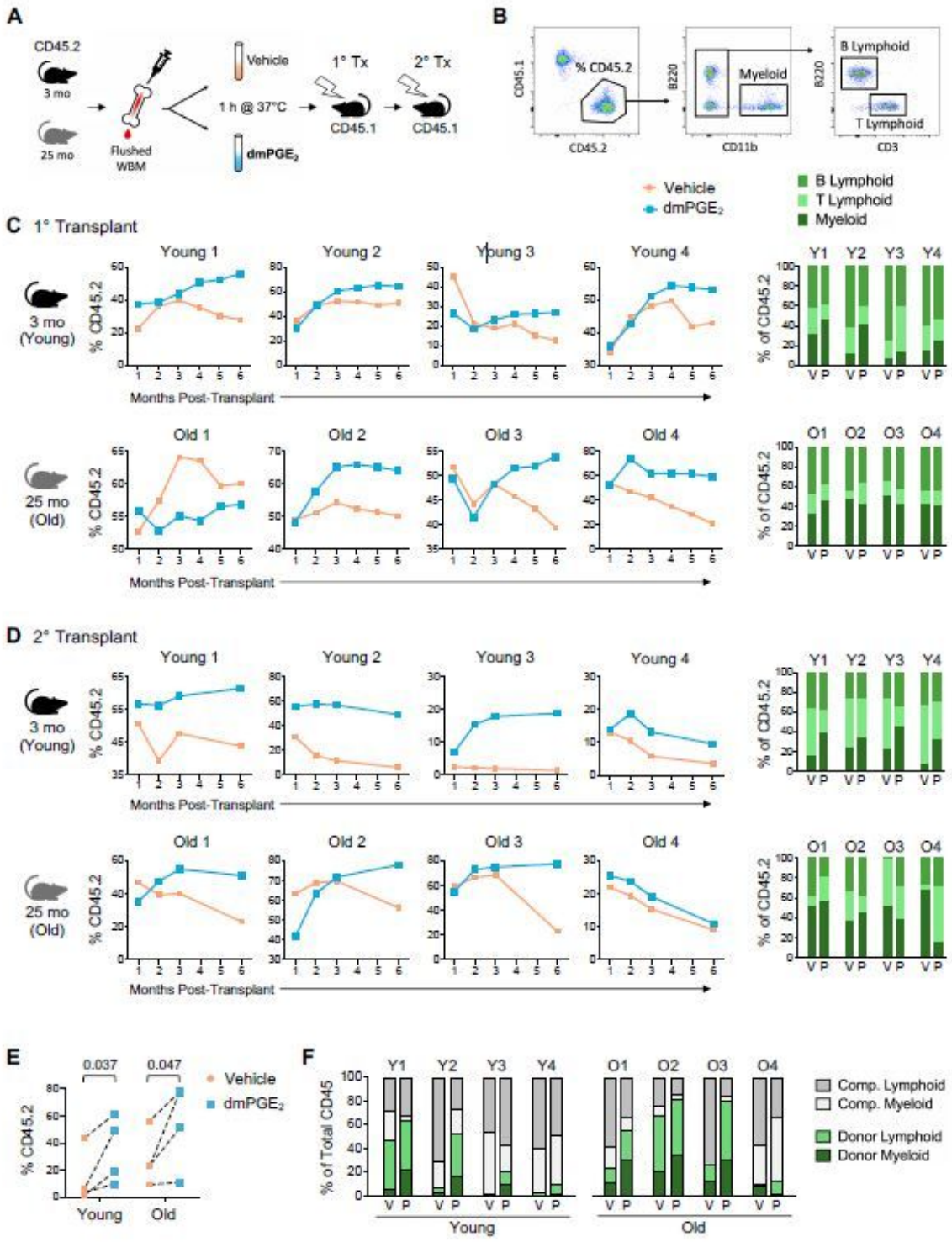

Figure 1

DmPGE2 pulse increases long-term chimerism in serial transplantation of old BM similar to young. A) WBM cells from 4 young (3 mo) and 4 old (25 mo) mice expressing CD45.2 were pulsed ex vivo for 1 h with dmPGE2 or vehicle, and 3 x 105 cells transplanted along with 2 x 105 untreated congenic (CD45.1) competitor WBM cells into irradiated CD45.1 primary (1°) recipients (3 recipients per treatment per donor = 12 recipients per group). After 7 mo, 1.5 x 106 WBM cells from 1° recipients were transplanted into

irradiated secondary (2°) recipients; Tx, transplant. B) Recipient PB was analyzed monthly for CD45.2 chimerism and trilineage reconstitution at 6 mo by flow cytometry as shown. C) 1° and D) 2° donor-derived chimerism over time for 4 young (Young 1-4) and 4 old (Old 1-4) donors when pulsed with either vehicle or dmPGE2 (left), and trilineage ratios within the CD45.2 compartment (right) at 6 mo post-transplant; V, vehicle; P, dmPGE2. E-F) Summary of young and old 2° transplant donor chimerism at 6 mo, showing E) the change in CD45.2 chimerism of each graft after dmPGE2 pulse, and F) donor and competitor lymphoid and myeloid populations as a frequency of total CD45 cells (CD45.1 + CD45.2), normalized to 100%; V, vehicle; P, dmPGE2. P-values from paired t-tests are indicated in (E).

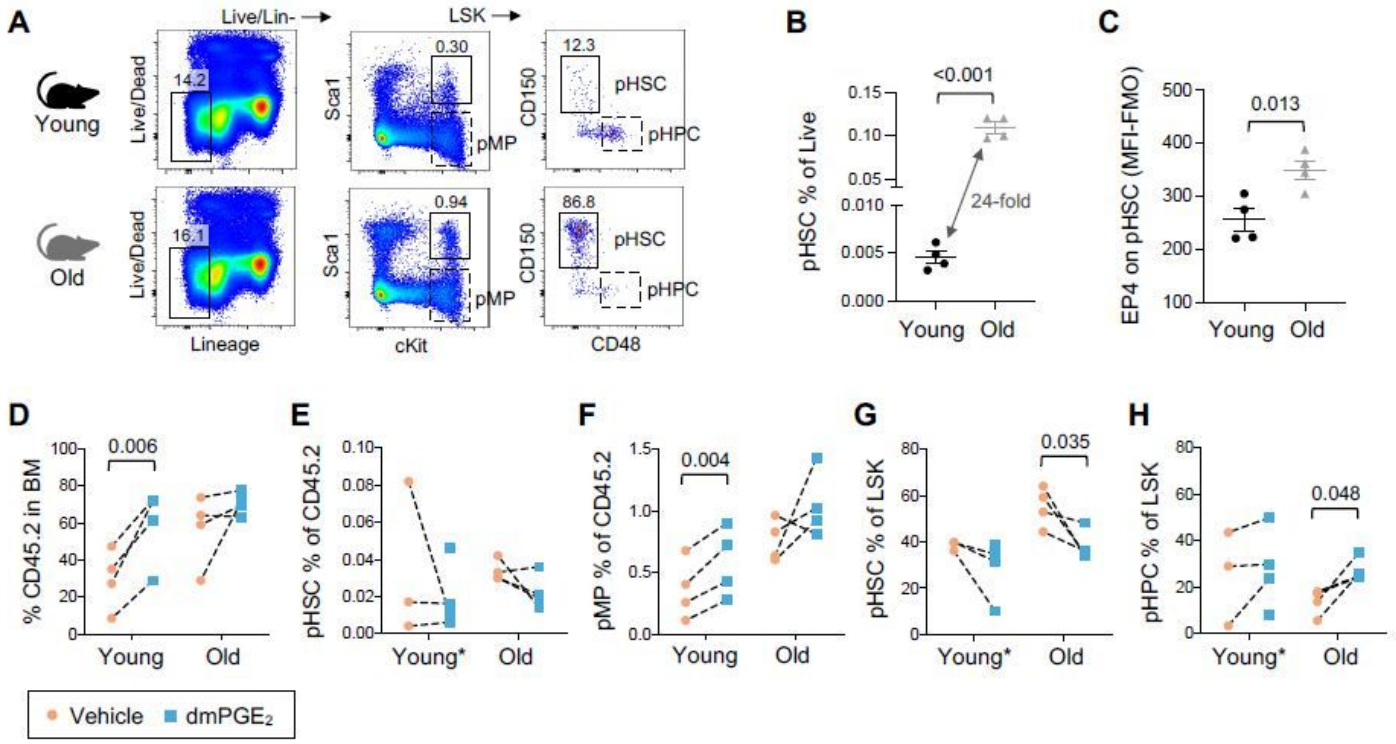

Figure 2

BM population analysis pre- and post-transplantation. A) Flow cytometry gating strategy for phenotypic analysis of BM cells; representative pre-pulse young and old BM samples are shown. B) Frequency of phenotypic HSCs (pHSCs) among live BM cells, and C) EP4 surface expression, for each young and old donor sample prior to pulse and transplantation; p-values from paired t-tests (2-tailed) are indicated on graphs. D-H) Upon harvest for 2° transplant (7 mo following 1° transplant) recipient BM was analyzed by flow cytometry for D) donor CD45.2 BM chimerism, E) pHSC and F) phenotypic myeloid progenitor (pMP) frequency of donor cells, and G) pHSC and H) pHPC frequency of the donor LSK population; shown with paired vehicle and dmPGE2 values connected by the dotted line; p-values from paired t-tests (1-tailed) are indicated on graphs. pHSC, phenotypic hematopoietic stem cells; pMP, phenotypic myeloid progenitors; pHPC, phenotypic hematopoietic progenitor cells. *Primary recipients of vehicle-treated BM from donor

mouse “Young 3” had too few CD45.2 LSK cells detected for pHSC/HPC frequencies, and were thus not included in graphs.

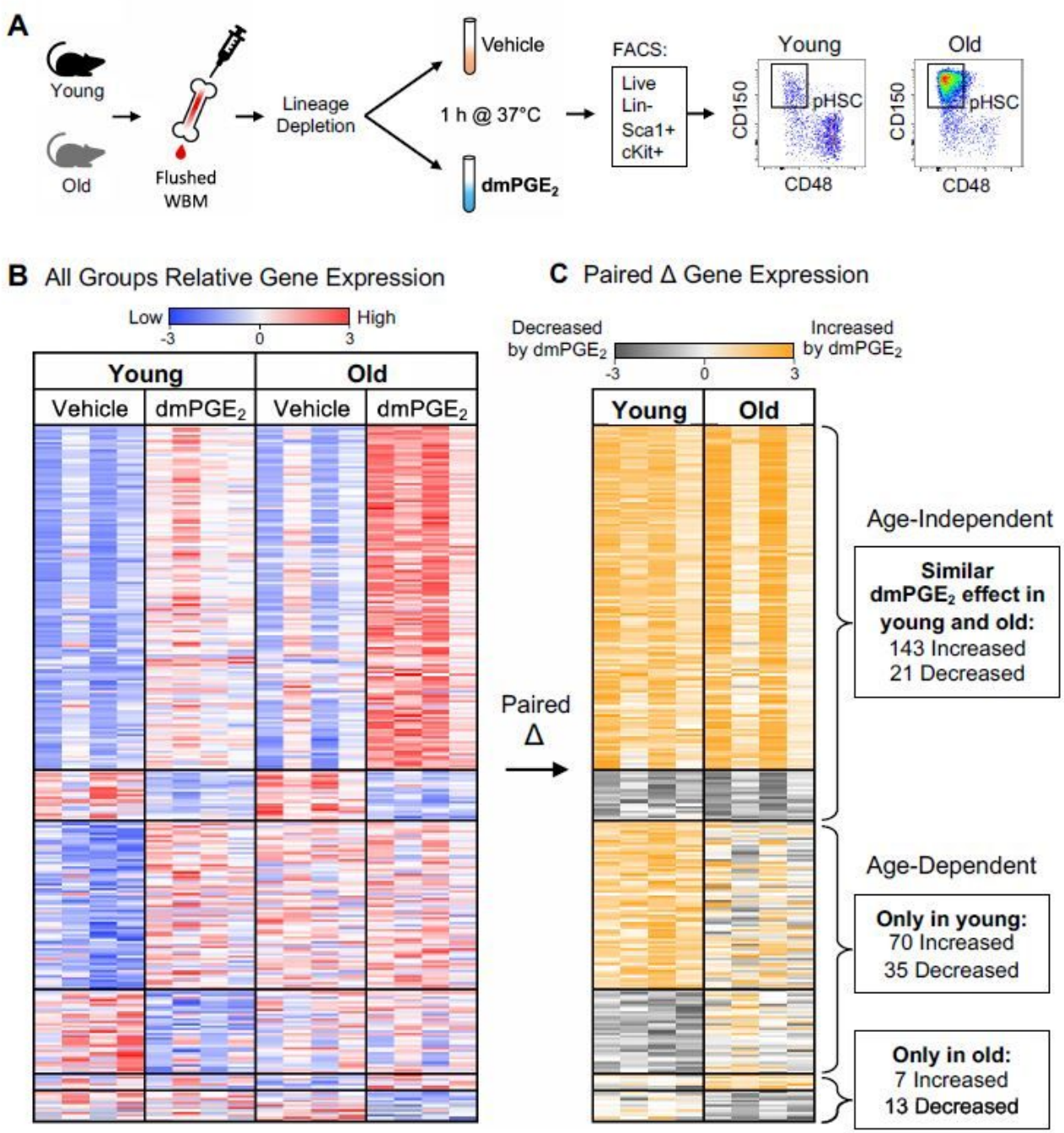

Figure 3

Transcriptional responses to dmPGE2 in young and old HSCs by RNA-seq. A) WBM cells from young (3 mo) and old (25 mo) mice were enriched for immature cells by magnetic lineage depletion then pulsed for 1 h with dmPGE2 or vehicle prior to staining and FACS sorting of pHSCs for RNA-seq. B) Relative expression heatmap and C) Paired Δ (change with dmPGE2 vs. vehicle) heatmap of all 289 genes with FDR<0.05 for dmPGE2 vs. vehicle in young and/or old pHSCs combined. Genes are clustered by dmPGE2

effects that are age-independent (same Δ direction and FDR<0.6 in other age group) or age-dependent (FDR>0.6 in other age group).

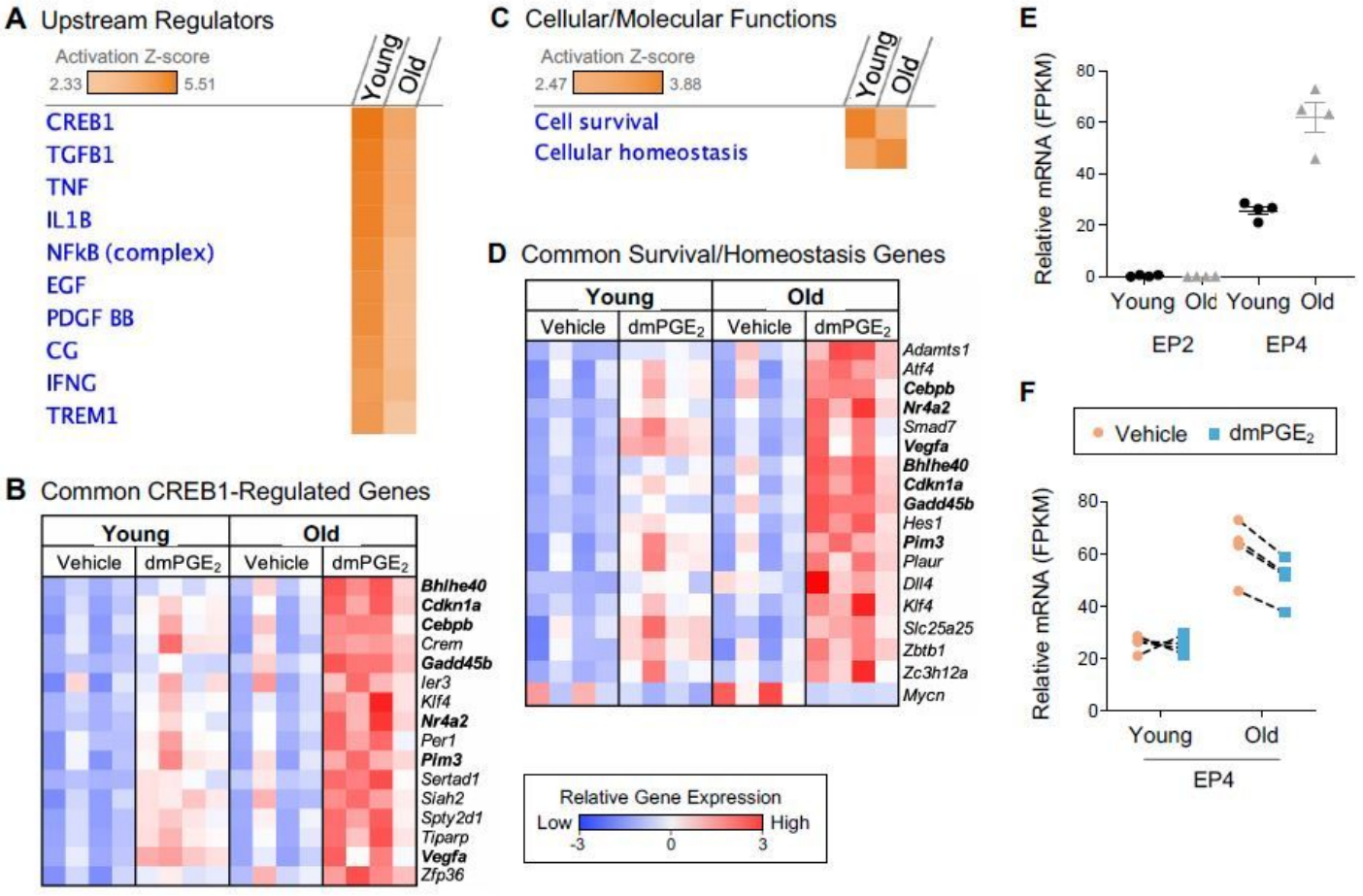

Figure 4

Bioinformatic comparison indicates preserved dmPGE2 signaling pathways in old HSCs. A) Top 10 Upstream Regulators by z-score from an IPA Comparison Analysis between the young and old dmPGE2 vs. vehicle analyses (z-score cutoff > |2|). B) Genes contributing to the CREB1 activation z-score in both young and old analyses. C) Cellular/Molecular Functions by z-score from the Comparison Analysis as in (A) (z-score cutoff > |2|). D) Genes contributing to the 'Cell survival' and/or 'Cellular homeostasis' activation z-scores in both young and old analyses. Bold genes in (B) and (D) are common in both heatmaps. E-F) Relative mRNA levels detected for PGE2 receptors E) EP2 and EP4 from vehicle-treated groups (baseline), and F) EP4 with paired vehicle and dmPGE2 values connected; FPKM, fragments per kilobase per million mapped reads.

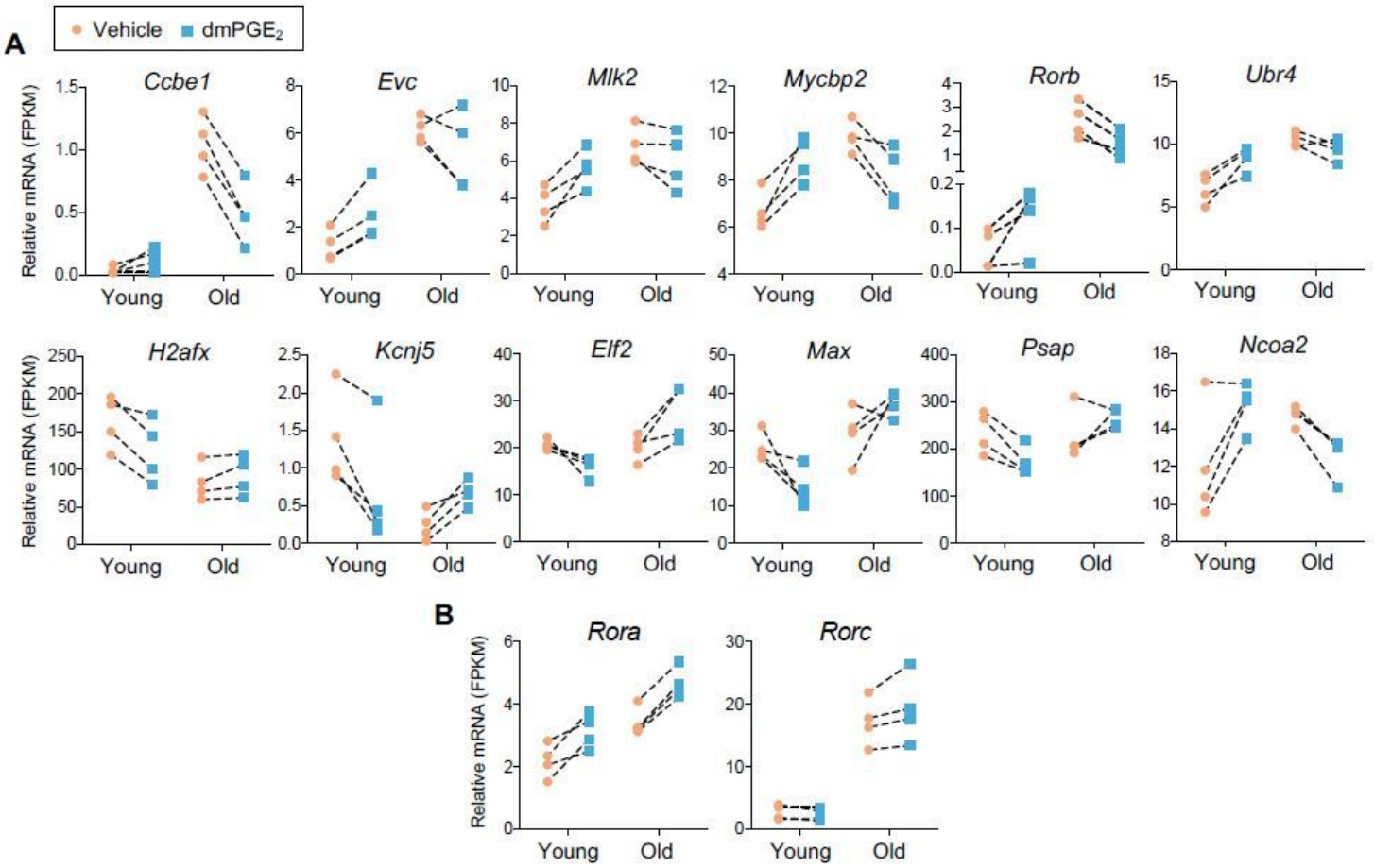

Figure 5

Genes oppositely affected by dmPGE2 stimulation in young and old HSCs. Relative mRNA levels are shown for each gene in young or old HSCs with paired vehicle and dmPGE2 values connected, for A) twelve genes identified as significantly differently affected by dmPGE2 based on “difference of difference” FDRs (all FDR<0.05), and B) other Rorb family members for comparison; FPKM, fragments per kilobase per million mapped reads.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GraphicalAbstract.pdf
- AgedPGEPulseFigS1.pdf