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Original Research

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Leukemia Inhibitory Factor Promotes Survival of Hematopoietic Progenitors Ex Vivo and Is Post-Translationally Regulated by DPP4

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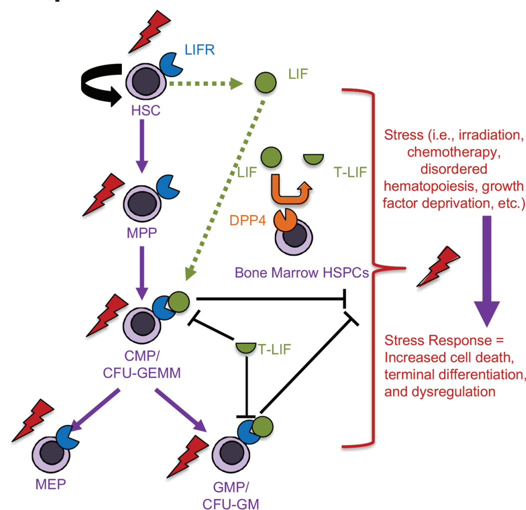
Abstract

Hematopoietic cells are regulated in part by extracellular cues from cytokines. Leukemia inhibitory factor (LIF) promotes survival, self-renewal, and pluripotency of mouse embryonic stem cells (mESC). While genetic deletion of LIF affects hematopoietic progenitor cells (HPCs), the direct effect of LIF protein exposure on HPC survival is not known. Furthermore, post-translational modifications (PTM) of LIF and their effects on its function have not been evaluated. We demonstrate that treatment with recombinant LIF preserves mouse and human HPC numbers in stressed conditions when growth factor addition is delayed ex vivo. We show that *Lif* is upregulated in response to irradiation-induced stress. We reveal novel PTM of LIF where it is cleaved twice by dipeptidyl peptidase 4 (DPP4) protease so that it loses its 4 N-terminal amino acids. This truncation of LIF down-modulates LIF's ability to preserve functional HPC numbers ex vivo following delayed growth factor addition. DPP4-truncated LIF blocks the ability of full-length LIF to preserve functional HPC numbers. This LIF role and its novel regulation by DPP4 have important implications for normal and stress hematopoiesis, as well as for other cellular contexts in which LIF and DPP4 are implicated.

Key words: hematopoiesis; hematopoietic progenitor cells; cytokine; leukemia inhibitory factor; serine proteases; post-translational modification.

Graphical Abstract



Significance Statement

This study is the first to examine the direct effects of Leukemia inhibitory factor protein on mouse and human hematopoietic progenitor cell survival in stressed conditions and the first to show that Leukemia inhibitory factor is post-translationally modified by Dipeptidyl peptidase 4. Leukemia inhibitory factor preserves hematopoietic progenitor cells during ex vivo stress, which is important in the context of dysregulated hematopoiesis or other stress conditions such as radiation. Modification by Dipeptidyl peptidase 4 down-modulates this pro-survival function. This novel axis of regulation could have important implications for normal and stressed hematopoiesis as well as other cellular contexts.

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Introduction

Blood cells are consistently replenished throughout an organism's life span.[1] Hematopoietic stem cells (HSCs) self-renew and respond to extracellular and intracellular cues to differentiate into lineage defined hematopoietic progenitor cells (HPCs), which in turn respond to extracellular and intracellular cues to differentiate into mature blood cells responsible for immune responses, wound healing, tissue oxygenation, and other essential functions.[2] HSCs/HPCs are regulated in part by extracellular protein-protein interactions between regulatory proteins known as cytokines and their associated cell surface receptor proteins.[3-6]

Leukemia inhibitory factor (LIF), initially discovered as a secreted protein that induces differentiation of a murine myeloid leukemia cell line in vitro,[7] promotes survival, self-renewal, and pluripotency of mouse embryonic stem cells (mESCs).[8,9] Murine (m) LIF is used as an essential factor to maintain pluripotent mESC and induced pluripotent stem cell (iPSC) cultures in ex vivo settings.[10] Human (h) LIF does not necessarily have similar essential functions in the maintenance of human ESC (hESCs), suggesting that LIF plays different regulatory roles in human and mouse cells.[11] Genetic whole-mouse deletion of LIF reduces pools of HPC-derived colony-forming units (CFUs).[12] A small phase I clinical trial showed that treatment of patients receiving chemotherapy with recombinant (r) hLIF yielded enhanced platelet recovery compared to patients receiving placebo.[13] However, there have not been follow-up clinical trials since the paper,[13] and direct effects of mLIF and hLIF on murine and human primitive hematopoietic cells have not been examined.

Post-translational modifications (PTM) are important mechanisms of regulation for many proteins, and such modifications of cytokines/chemokines dramatically affect their various functions.[14] One common mechanism by which cytokines may be post-translationally modified is via peptide cleavage by various proteases.[14] Dipeptidyl peptidase 4 (DPP4) is a serine protease that cleaves N-terminal dipeptides from proteins containing proline, alanine, or serine residues in the penultimate N-terminal location.[15-19] DPP4 is a cell surface anchored or soluble protein expressed by cells residing in the bone marrow (BM), including mouse and human hematopoietic cells.[20-22] DPP4 truncation of cytokines affects their functions. DPP4 is thus an important hematopoietic regulatory protein[15,23,24] that is sometimes ignored in studies of cytokine actions. We previously proposed that LIF contains a putative cleavage site for DPP4,[17,19] but this potential regulation of functional LIF has not been studied.

LIF and DPP4 play essential regulatory roles in a variety of cell types,[11,25-28] and are implicated in disease, in particular cancer.[29-32] However, interplay between these 2 proteins has not been explored and may be an important regulatory axis in different biological contexts. We sought to understand the roles of LIF in hematopoiesis. We showed that LIF receptor (LIFR) is expressed in mouse and human hematopoietic cells and demonstrated that LIF preserves functional HPC survival in the context of stressed hematopoiesis ex vivo. We reanalyzed publicly available sequencing data to show that *Lif*, genes encoding LIFRs, and LIF-associated signaling pathways were upregulated after whole-body irradiation of mice, an instance of stress hematopoiesis. We identified a novel PTM of LIF that ablates its function of preserving functional HPC survival ex vivo and demonstrated that DPP4-modified LIF down-modulates unmodified LIF function. This has implications for the role of LIF in normal, stressed, and disordered hematopoiesis, as well as in other cellular contexts.

Materials and Methods

Microarray Data Mining

Microarray data were mined from the BloodSpot database for the indicated gene expression profile. Data were plotted as log2 normalized expression values.[33]

Primary Cell Collection

Primary mBM cells were harvested by flushing femurs and/or tibiae. For flow cytometry analysis, mBM cells were lineage depleted using the Direct Lineage Depletion Kit (Milltenyi Biotec, Bergisch Gladbach, Germany). Human cord blood (CB) units were obtained from CryoCell (Oldsmar, FL) or Cleveland Cord Blood Center (Cleveland, OH). CB was processed within 48 hours of collection. Low-density hCB (LDCB) cells were obtained by washing CB once with phosphate-buffered saline (PBS) and layering CB on Ficoll-Paque Plus (GE Healthcare, Chicago, IL) to separate LDCB by centrifugation. LDCB was either used for assays or further enriched for CD34^{+} cells using the CD34^{+} Selection Kit (Milltenyi). All mouse studies were approved by the Indiana University School of Medicine (IUSM) IACUC. All CB studies were approved by the IUSM IRB.

Flow Cytometry

For LIFR cell surface expression, mBM or hLDCB enriched for CD34^{+} cells were stained using fluorochrome-conjugated antibodies targeting cell surface proteins used to immunophenotypically define HSCs/HPCs as well as anti-h/m LIFR antibody. Antibodies and cell surface markers used to define HSC/HPC populations are shown in Supplementary Tables 1 and 2. Populations examined for mLIFR expression were lineage negative (lin^{-}); lin-cKIT^{+}SCA1^{+} (LSK); lin-cKIT^{+}SCA1^{-} (LKS^{-}); long-term HSC (LT-HSC); short-term HSC (ST-HSC); multipotent progenitors (MPP); common myeloid progenitors (CMP); megakaryocyte/erythroid progenitors (MEP); and granulocyte/macrophage progenitors (GMP). Populations examined for hLIFR expression were CD34^{+}; CD34^{+}CD38^{-}; CD34^{+}CD38^{+}; HSC; MPP; multi-lymphoid progenitors (MLP), GMP; CMP; and MEP.

Sequence Alignment

Sequence alignment used UniProt database.[34]

Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry (LC-QTOF-MS)

RmLIF (BioLegend Cat# 554006) or rhLIF (BioLegend Cat# 593904) that are produced with N-terminal sequences with putative DPP4 truncation sites (not all recombinant LIF formulations have this putative DPP4 truncation site; Supplementary Table 3) were incubated with rhDPP4 at a ratio of 1 µg LIF:0.25 µg DPP4 at 37°C for 18 hours or indicated time points. 1-20 µL of protein mixture was injected on an LC-QTOF mass spectrometer (Agilent, Santa Clara, CA) using a C3 chromatography column. Data were analyzed using BioConfirm software to deconvolute protein species for identification.

HPC CFU Assay

After harvest, 2.5-5 × 10^4 mBM/hLDCB cells were plated, respectively, in the presence or absence of 50 ng/mL rmLIF/rhLIF, DPP4-truncated rmLIF/rhLIF, or a combination of full-length and truncated rmLIF/rhLIF in either 0.3% agar or 1% methylcellulose culture medium. Cells received different combinations of recombinant growth-stimulating factors on day of plating (day 0) or not until the subsequent day (day 1). Growth factors were 10 ng/mL granuloctye/macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Minneapolis, MN) alone; 10 ng/mL GM-CSF + 50 ng/mL stem cell factor (SCF) (R&D Systems); for mouse only 10 ng/mL rmGM-CSF + 50 ng/mL rmSCF + 5% v/v pokeweed mitogen mouse spleen cell-conditioned medium (PWMSCM) + 0.1 mM Hemin; for human only 10 ng/mL rhGM-CSF + 50 ng/mL rhSCF + 50 ng/mL rh erythropoietin (EPO) (Amgen, Thousand Oaks, CA) + 10 ng/mL rh interleukin 3 (IL-3) (R&D Systems). Mouse/human recombinant cytokines were used and for mouse/human assays, respectively. Plates were incubated in a humidified atmosphere of 5% CO_2 and lowered (5%) O_2.[23,35] Plates were scored for CFU-granulocyte/macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E) (for mBM only), and CFU-granulocyte/erythrocyte/monocyte/megakaryocyte (CFU-GEMM) progenitors after 6-7 days (mouse) or 13-14 days (human). Please note that this combinations of growth factors does not pick up hCB BFU-E, but rather only CFU-GEMM of erythroid containing colonies.[36]

Analysis of Publicly Available RNA-seq Data

Raw count matrices for RNA-seq of HSCs isolated from irradiated or non-irradiated mice were downloaded from the Gene Expression Omnibus dataset GSE151799.[37] Eight replicates were used for non-irradiated or irradiated (1 hour post-8.53 Gy whole-body irradiation) treatment groups. Differential expression analysis was performed using DESeq2.[38] Reported count values were normalized by library size. Reported significance is based on adjusted *P* values. Fast Gene Set Enrichment Analysis (FGSEA) was performed using the test statistic value to rank genes by differential expression confidence.[39]

Statistical Analysis

For CFU assays, n = 2-5 where each n is a biological replicate representing cells from different mice or CB units. Each biological replicate is made up of 2-3 technical replicates. Two-way ANOVA was performed to assess differences in treatment effect (PBS vs LIF) and time of growth factor addition (day 0 vs day 1). Data points were matched by mouse or by CB unit for both effects. Sphericity and normality of the sampled populations were assumed. Sidak's post hoc test was used for pairwise comparisons between all groups and *P* values from these tests were reported.

Results

LIFR Expression on Mouse and Human HSC/HPC

To determine whether LIF may have direct effects on HSCs/HPCs by binding to its known receptor, LIFR, we first examined whether LIFR is expressed on human and mouse HSC/HPC surfaces. We used the publicly available RNA microarray data compiled by the BloodSpot database to look at the transcript expression of *Lifr*.[33] *Lifr* RNA is expressed at detectable levels in mHSCs/HPCs including LT-HSC, ST-HSC, and various primitive progenitor cell types (Fig. 1A). We used flow cytometry to examine LIFR cell surface expression of various immunophenotypically defined mHSCs/HPCs. LIFR is expressed on 4.2%-5.8% of mBM cells. Within mBM, LIFR is expressed most highly on LT-HSCs (9.9%-26.7%) and MPPs (6.4%-43.2%) (Fig. 1B). It is expressed on low to moderate numbers of ST-HSC (6.4%-14.4%) and CMPs (3.9%-25.7%) and is expressed on MEPs (3.5%-13.9%) and GMPs (0.1%-5.7%) (Fig. 1B). BloodSpot data revealed *LIFR* RNA expression at detectable levels in various human hematopoietic cells, including HSCs and early progenitors (Fig. 1C). LIFR is expressed on cell surfaces of hCB HSCs/HPCs subpopulations, although its expression is apparent on relatively lower numbers of these cells (Fig. 1D). In LDCB enriched for $CD34^+$ cells, 0.3%-2.9% of cells express cell surface LIFR. The highest percentage of $LIFR^+$ cells are observed in MLPs (1.1%-11.9%) and HSCs (0.4%-3.4%), with lower percentages in GMPs (0.1%-1.6%), CMPs and MEPs (combined subpopulation CMP/MEP 0.2%-1.4%) populations and is essentially undetectable in MPPs (Fig. 1D). BloodSpot microarray data show that *IL6ST* (GP130), which codes for the other receptor essential for LIF binding and signaling, is also expressed in mouse and human HSCs/HPCs (data not shown).[33] This suggests that low to moderate numbers of mouse and human HPCs may be capable of LIF-dependent signaling via LIFR cell surface receptor.

LIF Enhances Mouse HPC CFU Numbers after Delayed Growth Factor Addition

Whole-body genetic deletion of murine *Lif* leads to reduced pools of HPCs in vivo.[12] We, therefore, evaluated whether LIF protein can promote the preservation of functional HPCs by colony formation ex vivo. We first observed that the addition of rmLIF had no effect when it was added concurrently with growth factors in mouse BM CFU assays (Fig. 2A-2D). We therefore plated mouse BM cells in semi-solid methylcellulose or agar in the presence or absence of rmLIF with growth factors at day 0 or delayed addition of different growth factor combinations until day 1. Delayed addition of growth factors induces cellular stress and reduces CFU numbers, an ex vivo measurement of functional HPC survival. This allows examination of whether LIF treatment enhances HPC survival in stressed conditions.[23] Growth factor combinations used were (1) GM-CSF alone (stimulates colony formation of more mature CFU-GM); (2) GM-CSF + SCF (stimulates colony formation of more immature CFU-GM); and (3) PWMSCM + EPO + SCF (stimulates colony formation of more immature CFU-GM). Treatment with LIF on day 0 preserved an average of 59.6%, 41.3%, and 74.5% of CFU-GM after delayed addition of GM-CSF, GM-CSF + SCF addition, or PWMSCM + SCF + EPO + Hemin, respectively, compared to adding growth factors on day 0 (Fig. 2A-2D). LIF treatment yielded an average 1.42-fold increase, 1.56-fold increase, and 1.40-fold increase in CFU-GM after delayed addition of GM-CSF, GM-CSF + SCF, or PWMSCM + SCF+ EPO + Hemin, respectively, compared to PBS control (Fig. 2A-2D). Similarly, we examined whether LIF protein could enhance functional human HPC numbers after delayed growth factor addition. We plated human LDCB in methylcellulose or agar in the presence or absence of rhLIF. LIF treatment trended toward

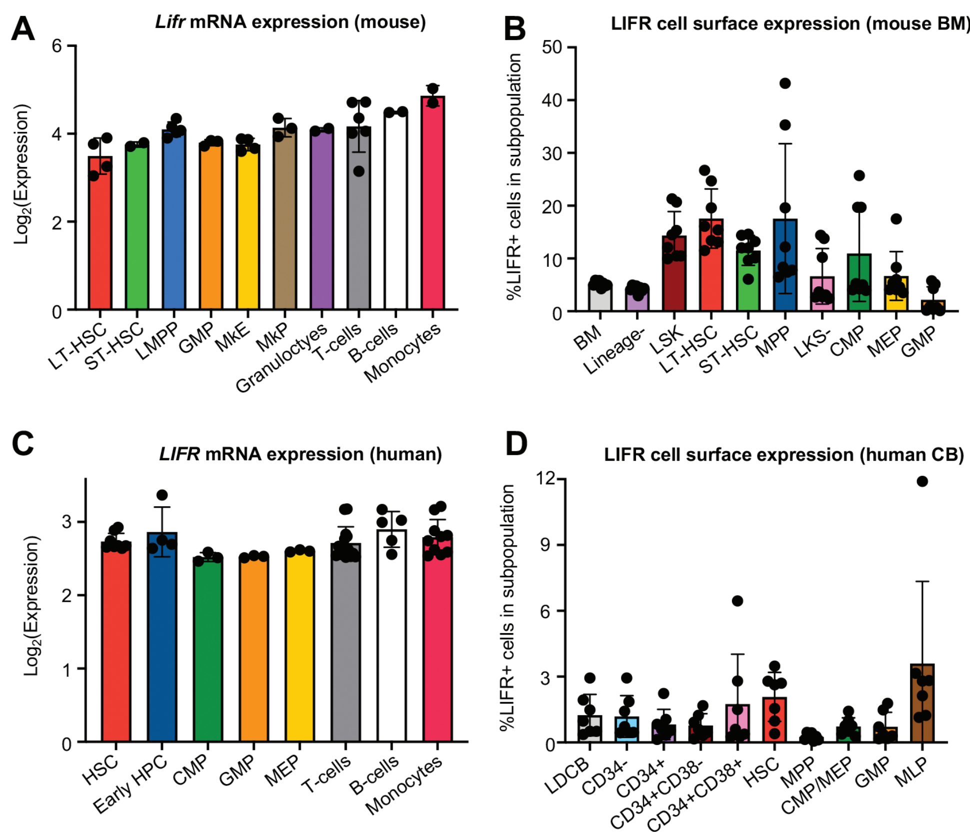


Figure 1. LIFR is expressed on the cell surface of primitive and mature mouse and human HPCs. (A, C) Micrarray data were mined for Lifr/LIFR expression in normal hematopoietic cells from the BloodSpot database.[33] (B, D) Cells were stringently immunophenotyped and were examined for LIFR cell surface expression by cell surface staining followed by flow cytometry (n = 7). LIFR^{+} gates were set based on an isotype control. Abbreviations: HPCs, hematopoietic progenitor cells; LIFR, leukemia inhibitory factor receptor; LMPP, lymphoid-primed multipotential progenitors; MkE, megakaryocyte/erythroid precursors; MkP, megakaryocyte precursor.

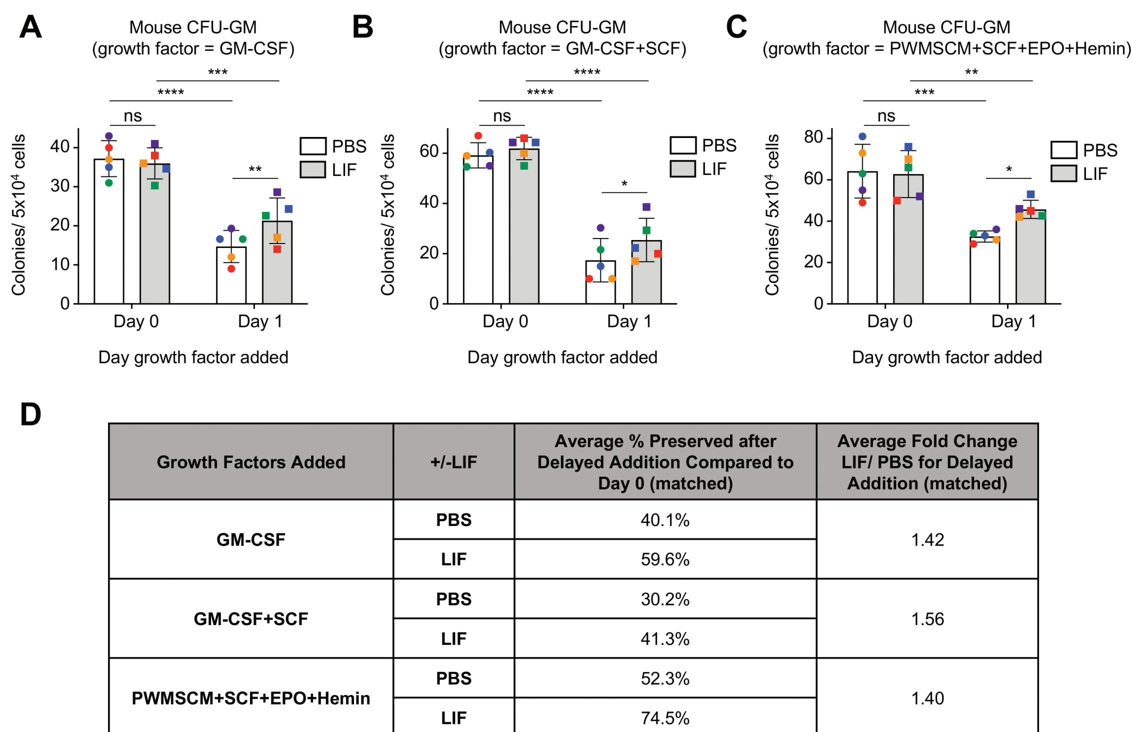
the same effects in human CB cells as in mouse BM, where treatment with LIF on day 0 preserved an average of 80.8% and 85.5% of CFU-GM after delayed addition of GM-CSF or GM-CSF + SCF addition, respectively, compared to adding growth factors on day 0 (Supplementary Fig. 1A-1C). LIF treatment yielded an average 1.52-fold increase and 2.1-fold increase after delayed addition of GM-CSF or GM-CSF + SCF, respectively, compared to PBS control (Supplementary Fig. 1A-1C). These data demonstrate that LIF significantly enhances HPC CFU numbers after delayed growth factor addition in mouse BM and may have similar effects in human CB.

LIF and LIF-Associated Genes Are Upregulated after Whole-Body Irradiation in Mice

Because LIF treatment on day 0 had no effect on functional HPCs but did have significant enhancing effects on functional HPC numbers with delayed growth factor addition, we speculate that LIF may function as a pro-survival cytokine for hematopoietic cells during stress hematopoiesis. Therefore, it is possible that LIF expression is regulated by stress responses. To examine this, we mined raw data from a publicly available dataset where HSCs were isolated for RNA-sequencing (RNA-seq) 1 hour following whole-body irradiation.[37] We reanalyzed these data and examined normalized expression levels of *Lif* and genes encoding LIFRs. Remarkably, *Lif* is upregulated more than 39-fold 1 hour after irradiation (Fig. 3A). We next examined the genes that encode the receptors required for LIF-induced signaling. *Lifr* trended toward being upregulated by irradiation, showing a 2-fold increase (Fig. 3B), and *Gp130* was significantly upregulated, showing a muted but more consistent 1.3-fold upregulation after irradiation (Fig. 3C). Thus, mouse BM cells (specifically HSCs), upregulate genes necessary for LIF signaling as a response to irradiation. We assessed by gene set analysis whether LIF-associated signaling pathways are altered after irradiation and found that genes upregulated by LIF treatment are upregulated after irradiation (Fig. 3D).[40] Similarly, signaling pathways that are closely associated with being induced by LIF in embryonic stem cells,[41] including PI3K-AKT signaling, STAT3-regulated genes, and MAPK signaling pathway, are significantly upregulated after irradiation (Fig. 3E-3G).[42] This signaling induction suggests that not only is LIF being upregulated on a transcriptional level, but that LIF might be secreted in the BM as a stress response and is, in turn, inducing changes in transcription. Taken together with its preservation of functional HPC following delayed growth factor addition ex vivo, these data suggest that LIF acts as a pro-survival molecule by inducing signaling during stress hematopoiesis, such as post-irradiation. We next pursued these findings in the context of potential PTM (by DPP4) on LIF.

LIF Contains Putative DPP4 Truncation Sites

The first species of mLIF that was discovered and isolated was confirmed by peptide sequencing to have an N-terminal sequence of PLPITPV and subsequent analysis of rhLIF suggested an identical N-terminal sequence.[43,44] However,



Growth Factors Added	+/-LIF	Average % Preserved after Delayed Addition Compared to Day 0 (matched)	Average Fold Change LIF/ PBS for Delayed Addition (matched)
GM-CSF	PBS	40.1%	1.42
	LIF	59.6%	
GM-CSF+SCF	PBS	30.2%	1.56
	LIF	41.3%	
PWMSCM+SCF+EPO+Hemin	PBS	52.3%	1.40
	LIF	74.5%	

Figure 2. mLIF preserves functional mouse HPC after delayed growth factor addition. (A-C) Mouse BM cells were isolated and treated ±50 ng/mL rmLIF on day 0. Indicated growth factors were added on day 0 or day 1 and CFU-GM scored 6 days after growth factor addition. Data points are colored to correspond to matched values from a biological replicate (cells isolated from the same mouse). n = 5 biological replicates (2-3 technical replicates each). Two-way ANOVA matched for treatment and day growth factor added with Tukey's post hoc testing. (D) Quantification of A-C. Shown are the average amounts of CFU-GM preserved when growth factors are delayed compared to when they are added at plating, matched for biological replicate and treatment. Shown are the average fold-changes of CFU-GM for LIF-treated cells compared to PBS-treated cells after delayed growth factor addition, matched for biological replicate. *P < .05; **P < .01; ***P < .001; ****P < .0001; ns, not significant (P > .05). Abbreviations: ANOVA, analysis of variance; BM, bone marrow; CFU-GM, colony-forming unit-granulocyte/macrophage; HPCs, hematopoietic progenitor cells; LIF, leukemia inhibitory factor; PBS, phosphate-buffered saline.

peptide sequence analysis of mLIF and hLIF has since revealed species of mLIF and hLIF that have the N-terminal sequence of SPLPITPV,[45,46] a potential DPP4 truncatable sequence. Interestingly, the latter sequence contains 2 putative cleavage sites for the protease DPP4: **SPLPITPV**, while the former is likely not a potential substrate of DPP4 (Fig. 4A). Further examination of full protein sequences of hLIF and mLIF was performed. Sequence alignment revealed that within the LIF signal peptide, mLIF contains an additional leucine residue compared to hLIF (Fig. 4B). The addition of this hydrophobic residue may affect the location of the cleavage site for the signal peptide, as the hydrophobic core of the signal peptide is a key determinant for signal peptide cleavage,[47,48] suggesting that mLIF may be more likely to exist as a non-DPP4-truncatable species. It is noteworthy that the hLIF isoform identified to have the putative DPP4 truncation sequence was a natural endogenous product from a cell line, while the mLIF isoform was expressed using an exogenous construct. Thus, multiple species of mLIF and hLIF exist in nature and this may be dependent on the cell types they are produced in. At least 1 identified sequence of each contains putative DPP4 truncation sites (Fig. 4A).

LIF Is Truncated by DPP4 In Vitro

To test whether hLIF and mLIF can be cleaved by DPP4, we incubated rhLIF or rmLIF with rhDPP4 protein and analyzed sizes of the protein by LC-QTOF-MS. By using LC-QTOF, we observed a shift in the size of hLIF and mLIF after incubation with DPP4 consistent with loss of their 4 N-terminal amino acids, indicating that DPP4 cleaved LIF twice (Fig. 4C, 4D). Thus, hLIF and mLIF are DPP4-truncatable, suggesting a possible role for DPP4 regulation of LIF-dependent function. The catalytic mechanism of DPP4 suggests that DPP4-dependent loss of 4 amino acids from LIF occurs by 2 sequential DPP4 truncations, confirmed by a time course experiment where mLIF was incubated with DPP4 for varying time intervals, up to and including 24 hours. A protein product with a size consistent with LIF that has lost its first 2 amine-terminal amino acids was identified within 15 minutes of exposure to DPP4. By 30 minutes, untruncated full-length LIF (FL-LIF) is a minor species in the mixture, while singly truncated LIF (T1-LIF) is the primary species, and a new protein product with a size consistent with LIF that has lost its 4 amine-terminal amino acids is identifiable. At 60 minutes after first exposure to DPP4, doubly truncated LIF (T2-LIF, herein referred to as T-LIF) is the only identifiable LIF protein species (Fig. 4E). To determine whether loss of the 4 N-terminal amino acids may have an effect on the structure/function of LIF, we performed a simple disorder prediction[49] for hFL-LIF and hT-LIF. T-LIF was predicted to be more disordered in all regions of its polypeptide sequence than FL-LIF (Fig. 4F), suggesting that T-LIF may be structured differently than FL-LIF, which could affect protein binding, stability, and function.

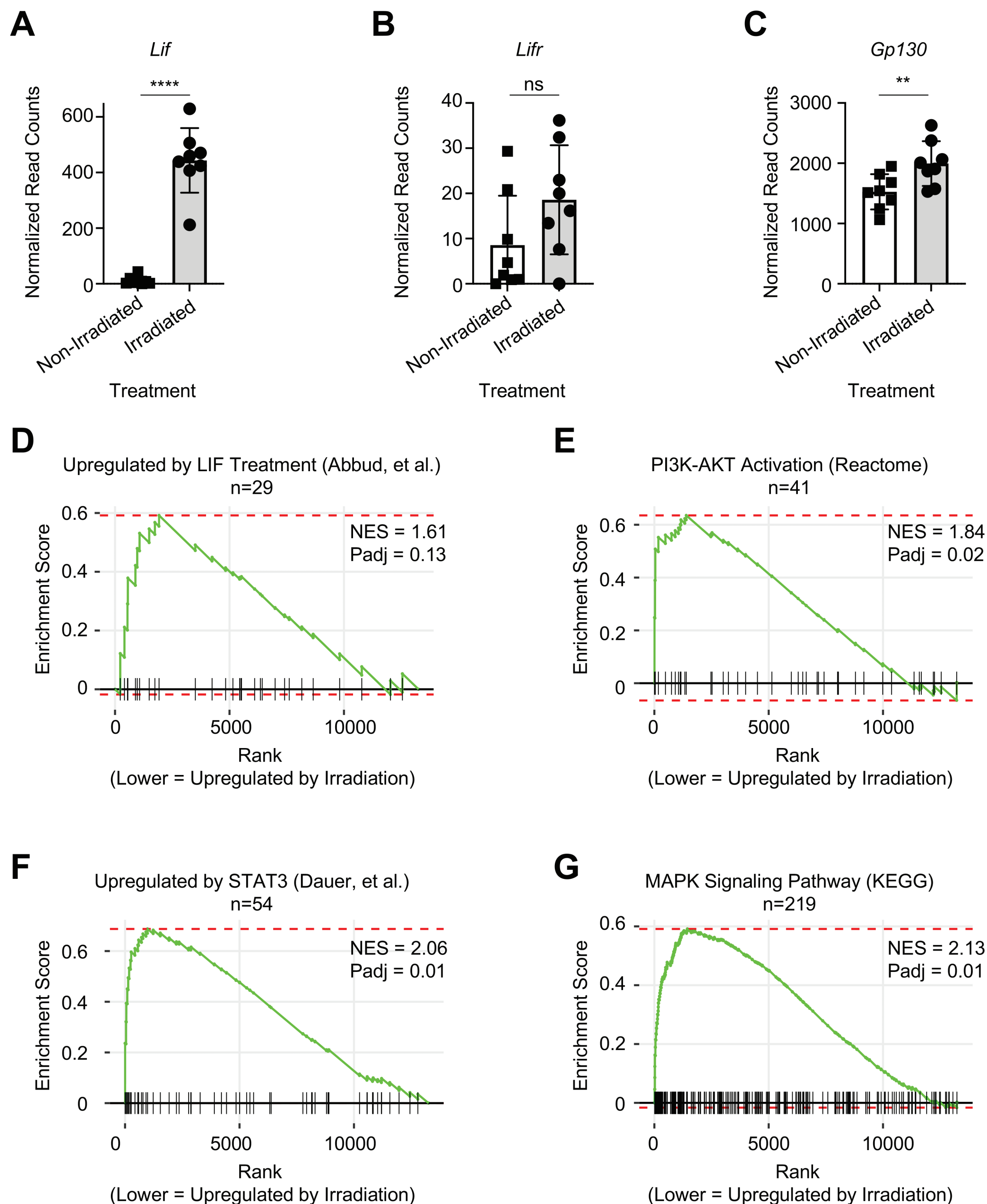


Figure 3. Mouse BM HSCs upregulate *Lif* and LIF-associated signaling as a response to irradiation. (A-G) Raw count matrices of RNA-seq data from BM HSCs isolated from mice after irradiation were downloaded from GSE151799[37] and reanalyzed using the DESeq2 R package. (A-C) Shown are read counts normalized by library size for the indicated genes for HSCs isolated from non-irradiated or irradiated mice. Significance between treatments at the gene level was determined by DESeq2 modeling. ****$P_{adj} < .0001$; **$P_{adj} < .01$; ns, not significant ($P_{adj} > .05$). (D-G) FGSEA plots showing positive enrichment of the indicated previously defined gene sets for HSCs isolated after irradiation. Abbreviations: BM, bone marrow; FGSEA, Fast Gene Set Enrichment Analysis; HSCs, hematopoietic stem cells; LIF, leukemia inhibitory factor; NES, normalized enrichment score; P_{adj}, adjusted *P* value.

DPP4 Truncation of LIF Down-Modulates Its Ability to Enhance HPC CFU Survival Ex Vivo after Delayed Growth Factor Addition

To examine whether truncation of LIF affects its ability to preserve HPC CFU survival, we treated mouse BM ex vivo with FL-LIF, T-LIF, or a combination of FL-LIF + T-LIF. Growth factors were added concurrently or after a 1-day delay. LIF treatments did not affect colony formation of CFU-GM when growth factors were immediately added. However, after delayed addition of GM-CSF compared to immediate addition of GM-CSF, FL-LIF-treated mouse BM cells preserved 103% of CFU-GM compared to a significantly reduced 49.9% for vehicle-treated cells and 44.9% for truncated LIF (T-LIF)-treated cells (Fig. 5A, 5D). FL-LIF also preserved 71.2% of CFU-GM after delayed GM-CSF + SCF addition compared to a significantly reduced 31.3% for vehicle and 30.1% for

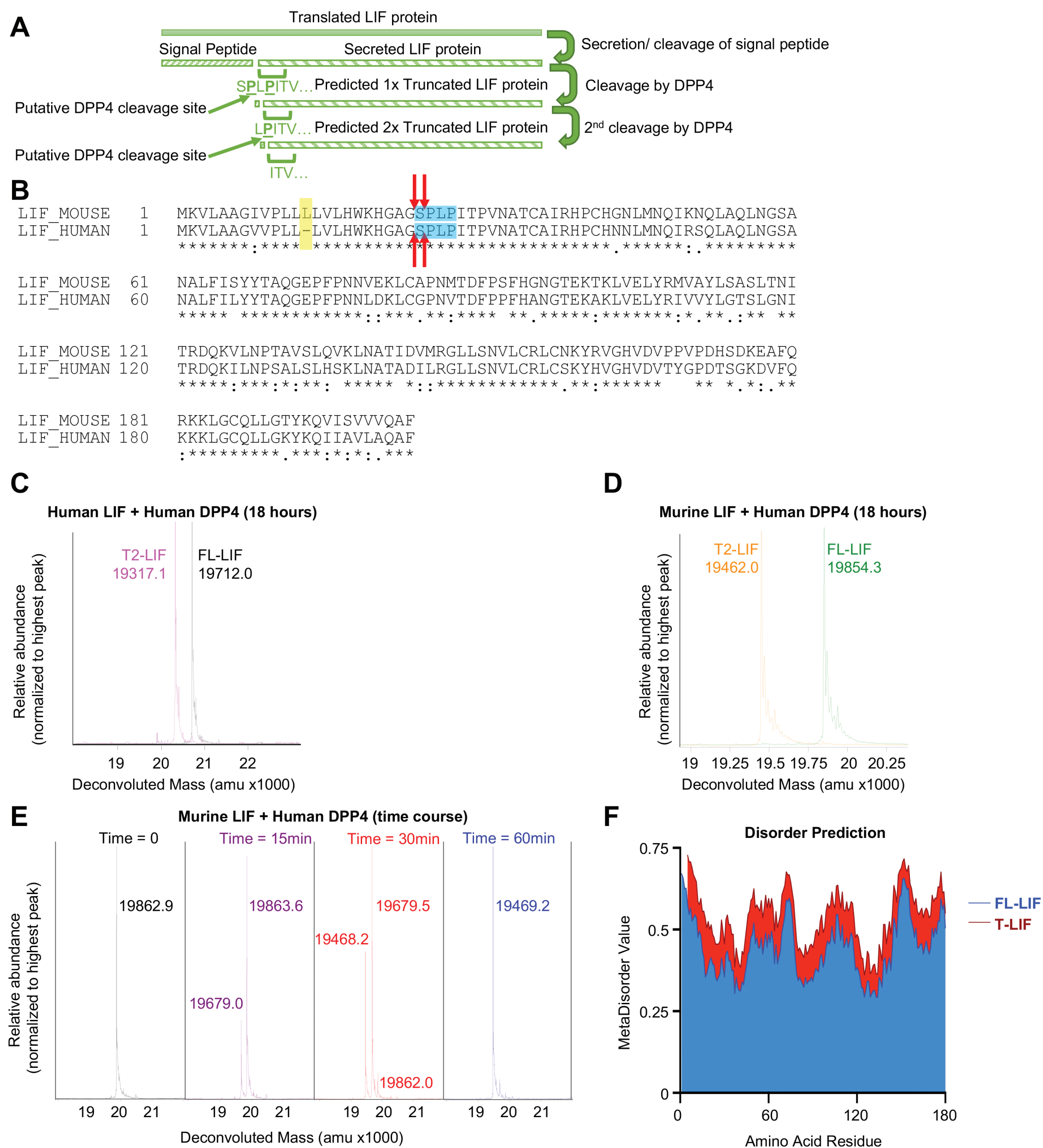
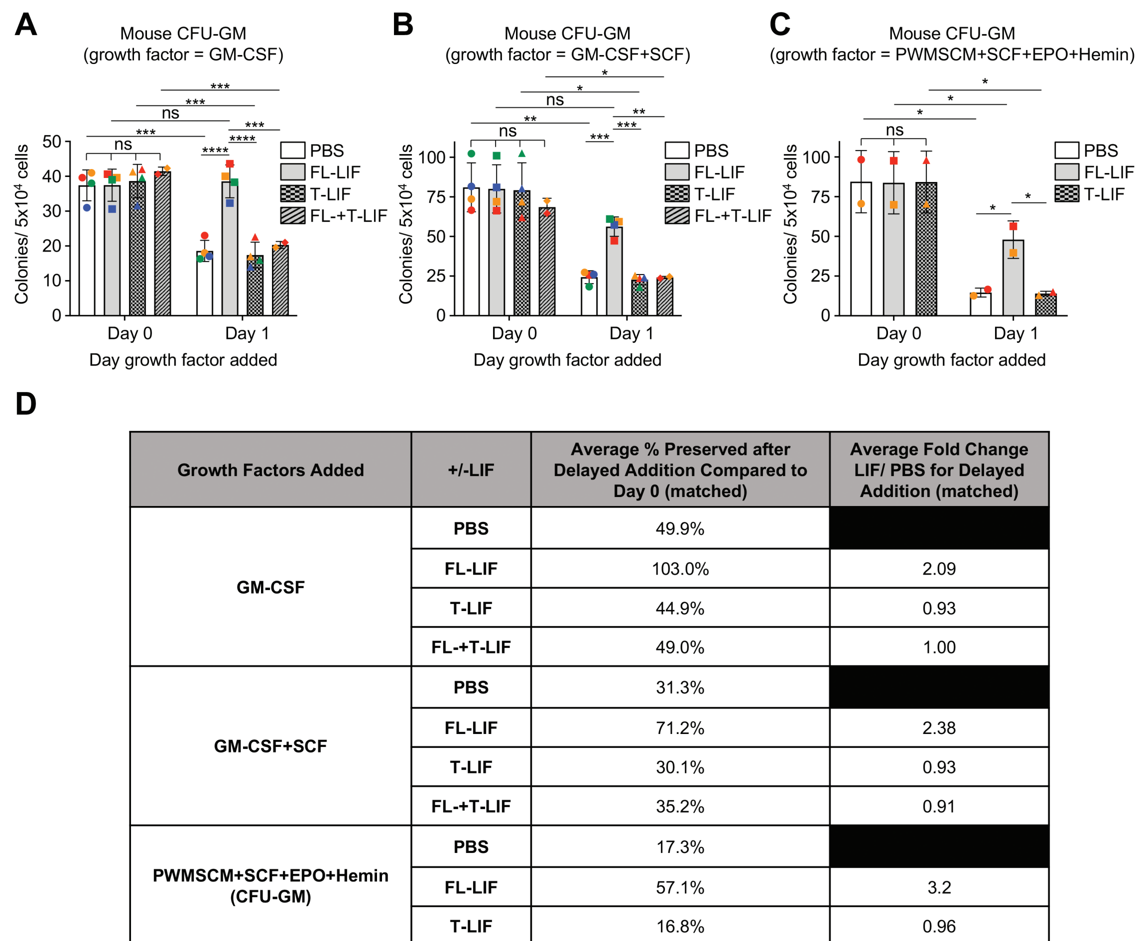


Figure 4. Human LIF is truncated twice by DPP4. (A) Proposed mechanism for post-translational modification of LIF by DPP4. (B) Sequence alignment of mLIF and hLIF. Yellow highlight indicates an additional hydrophobic residue found in the signal peptide of mLIF. Red arrows indicate the annotated signal peptide cleavage site. Blue highlight indicates the proposed tetrapeptide cleaved by DPP4. (C, D) hLIF or mLIF were incubated with rhDPP4 for 18 hours at 37°C, then analyzed by LC-QTOF for protein size inference. Graphs of DPP4-truncated LIF (T2-LIF) are overlaid with full-length LIF incubated with vehicle (FL-LIF). Shown are representative experiments that were performed >=2 times each. (E) Time course of DPP4 truncation of mouse LIF as determined by LC-QTOF. (F) Disorder prediction of human FL-LIF and T-LIF.[49] Abbreviations: DPP4, dipeptidyl peptidase 4; LC-QTOF, liquid chromatography quadrupole time-of-flight mass spectrometry; LIF, leukemia inhibitory factor.

T-LIF-treated cells (Fig. 5B, 5D). The addition of PWMSCM + EPO + SCF + hemin showed muted preservation effect for FL-LIF, where it preserved 57.1% of mCFU-GM, significantly lower than CFU-GM numbers when growth factors were added on day 0. This is still significantly more CFU-GM than vehicle or T-LIF, which only preserved 17.3% and 16.8% of CFU-GM, respectively (Fig. 5C, 5D). LIF treatment also trended toward preserving BFU-E and CFU-GEMM after delayed addition of PWMSCM + EPO + SCF + hemin, but the CFU numbers after delayed addition are too low to say this with statistical confidence (Supplementary Fig. 2A, 2B). Importantly, combination treatment with FL-LIF and T-LIF mimicked effects on HPC of T-LIF alone treatment after delayed growth factor addition in mouse cells (Fig. 5A, 5B, 5D). Thus, mT-LIF blocks the function of mFL-LIF to enhance the survival of CFU subjected to delayed growth factor addition.

We also examined whether human LIF truncation affects its ability to preserve functional HPC ex vivo. As with mBM, treatment with FL-LIF preserved colony-forming capacity of hCB HPC when GM-CSF + SCF addition is delayed, preserving 79% of CFU-GM. T-LIF loses the ability to preserve CFU-GM, retaining only 35.8% of CFU-GM, while vehicle treatment preserved 35.2% of CFU-GM (Fig. 6A, 6D). In delayed conditions of GM-CSF + SCF + EPO



Growth Factors Added	+/-LIF	Average % Preserved after Delayed Addition Compared to Day 0 (matched)	Average Fold Change LIF/ PBS for Delayed Addition (matched)
GM-CSF	PBS	49.9%	
	FL-LIF	103.0%	2.09
	T-LIF	44.9%	0.93
	FL-+T-LIF	49.0%	1.00
GM-CSF+SCF	PBS	31.3%	
	FL-LIF	71.2%	2.38
	T-LIF	30.1%	0.93
	FL-+T-LIF	35.2%	0.91
PWMSCM+SCF+EPO+Hemin (CFU-GM)	PBS	17.3%	
	FL-LIF	57.1%	3.2
	T-LIF	16.8%	0.96

Figure 5. Truncation of mLIF ablates its ability to preserve functional HPC. (A-C) Mouse BM cells were isolated and treated ±50 ng/mL FL-LIF, T-LIF, or FL-LIF + T-LIF on day 0. The indicated growth factors were added on day 0 or day 1, and CFU-GM was scored 6 days after growth factor addition. Data points are colored to correspond to matched values from a biological replicate (cells isolated from the same mouse). N = 2-4 biological replicates (2-3 technical replicates each). Two-way ANOVA matched for treatment and day growth factor added with Tukey's post hoc testing. (D) Quantification of A-C. Shown are the average amounts of CFU-GM preserved when growth factors are delayed compared to when they are added at plating, matched for biological replicate and treatment. Shown are the average fold-changes of CFU-GM for LIF-treated cells compared to PBS-treated cells after delayed growth factor addition, matched for biological replicate. $*P < .05$; $**P < .01$; $***P < .001$; $****P < .0001$; ns, not significant ($P > .05$). Abbreviations: ANOVA, analysis of variance; BM, bone marrow; CFU-GM, colony-forming unit-granulocyte/macrophage; HPCs, hematopoietic progenitor cells; LIF, leukemia inhibitory factor; PBS, phosphate-buffered saline.

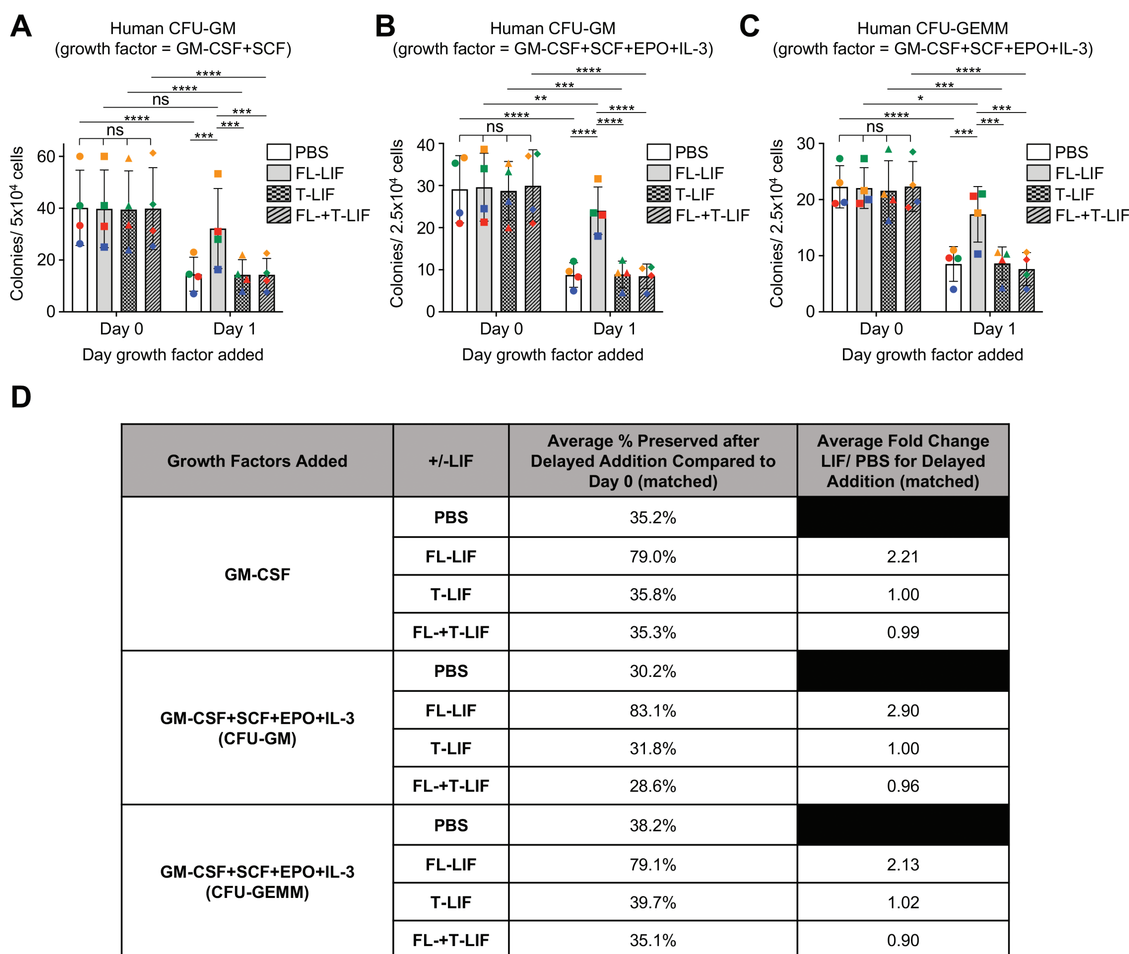
+ IL-3, FL-LIF preserves 83.1% of CFU-GM compared to significantly reduced 30.2% preservation for vehicle and 31.8% for T-LIF (Fig. 6B, 6D). In this same condition, FL-LIF also preserved 79.1% of the more primitive multipotential CFU-GEMM compared to a significantly reduced 38.2% preservation for vehicle and 39.7% preservation for T-LIF (Fig. 6C, 6D). As is the case with mHPC, T-LIF + FL-LIF treatment mimicked T-LIF alone treatment, suggesting that hT-LIF blocks hFL-LIF preservation of functional HPC (Fig. 6A-6D). Thus DPP4 truncation of LIF protein results in down-modulation of its ability to preserve functional mBM and hCB HPC survival ex vivo.

The degree to which LIF apparently preserves functional HPC numbers after delayed growth factor addition is variable, which is apparent when we examine the series of experiments in Fig. 2 and Supplementary Fig. 1 compared to the series of experiments performed in Figs. 5, 6. These differences may be caused by biological variability between the different samples, may be the result of differences in the health of the cells when isolated, or may be due to differences in the bioactivity of different lots of the purified recombinant LIF protein..

Discussion

We evaluated the role of LIF protein in preserving functional HPC survival ex vivo in stressed mBM and hCB. We also described a novel PTM of LIF by DPP4 that has profound effects on its function for HPC survival. These data have important implications for the role of LIF in hematopoietic regulation and more broadly for regulation of LIF in a wide range of contexts, yet to be determined.

We showed by data mining and cell surface expression analysis that receptors necessary for LIF to bind to for signal transduction are expressed on subsets of primitive and mature HPCs. Genetic depletion previously showed that whole-mouse LIF deletion reduces the numbers of HPC found in mBM.[12] We demonstrated the direct effects of LIF protein on early subsets of HPCs by examining functional HPC survival after delayed addition of growth factors ex vivo. LIF has no effect on HPC in the immediate presence of growth factors but preserves mouse and human CFU-GM/GEMM stimulated with various combinations of growth factors when the addition of these growth factors is delayed. This suggests that



Growth Factors Added	+/-LIF	Average % Preserved after Delayed Addition Compared to Day 0 (matched)	Average Fold Change LIF/ PBS for Delayed Addition (matched)
GM-CSF	PBS	35.2%	
	FL-LIF	79.0%	2.21
	T-LIF	35.8%	1.00
	FL-+T-LIF	35.3%	0.99
GM-CSF+SCF+EPO+IL-3 (CFU-GM)	PBS	30.2%	
	FL-LIF	83.1%	2.90
	T-LIF	31.8%	1.00
	FL-+T-LIF	28.6%	0.96
GM-CSF+SCF+EPO+IL-3 (CFU-GEMM)	PBS	38.2%	
	FL-LIF	79.1%	2.13
	T-LIF	39.7%	1.02
	FL-+T-LIF	35.1%	0.90

Figure 6. hLIF preserves functional HPC and truncation of hLIF ablates its ability to preserve functional HPC. (A-C) Human low-density CB was isolated and treated ±50 ng/mL FL-LIF, T-LIF, or FL-LIF + T-LIF on day 0. The indicated growth factors were added on day 0 or day 1 and CFU-GM or CFU-GEMM were scored 13 days after growth factor addition. As noted in Materials and Methods, CFU-GEMM but not BFU-E can be detected in hCB with the combinations of growth factors containing SCF and EPO.[36] Data points are colored to correspond to matched values from a biological replicate (cells isolated from the same CB unit). n = 4 biological replicates (2-3 technical replicates each). Two-way ANOVA matched for treatment and day growth factor added with Tukey's post hoc testing. (D) Quantification of A-C. Shown are the average amounts of CFU-GM/GEMM preserved when growth factors are delayed compared to when they are added at plating, matched for biological replicate and treatment. Shown are the average fold-changes of CFU-GM/GEMM for LIF-treated cells compared to PBS-treated cells after delayed growth factor addition, matched for biological replicate. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$; ns, not significant ($P > .05$). Abbreviations: ANOVA, analysis of variance; CB, cord blood; CFU-GEMM, colony-forming unit-granulocyte/erythrocyte/monocyte/megakaryocyte; CFU-GM, colony-forming unit-granulocyte/macrophage; EPO, erythropoietin; HPC, hematopoietic progenitor cells; LIF, leukemia inhibitory factor; PBS, phosphate-buffered saline; SCF, stem cell factor.

LIF functions to preserve functional HPC only when cells are stressed by deprivation of growth factors. Indeed, we found that whole-body irradiation of mice led to a rapid strong upregulation of *Lif* transcription and genes belonging to LIF-associated signaling pathways, as well as more muted but clear upregulations of genes encoding LIFRs, suggesting that LIF expression may be induced as a stress response for the induction of pro-survival signaling pathways. This could have important implications for the role of LIF in hematopoietic recovery after irradiation, transplantation, and disordered hematopoiesis. It is noteworthy that while we focused primarily on the direct effects of LIF on functional myeloid committed HPCs due to the availability of the CFU assay as a gold-standard assay to evaluate the effects of a purified protein on this subset of cells directly, the highest levels of LIFR were found on HSCs and MPPs in mBM and HSCs and MLPs in hCB. Thus, it is possible if not probable that earlier and more potent HSC/HPC as well as lymphoid progenitors are also affected by LIF treatment, but this must be studied further.

We demonstrated that DPP4 regulates a novel PTM of LIF by sequentially truncating 2 N-terminal dipeptides from the protein, with DPP4 causing loss of 4 LIF N-terminal amino acids. Unlike FL-LIF, T-LIF does not have the ability to preserve functional HPCs after delayed growth factor addition. Furthermore, T-LIF blocks the ability of FL-LIF to preserve HPC CFUs when they are both present, suggesting that LIF functions are regulated by DPP4. This might have broader implications for other cellular systems and for diseases where LIF and DPP4 both play a role, such as in various subtypes of cancer.[32,50-55] The PTM we identified may not occur for all isoforms of mLIF and hLIF. For example, endogenous hLIF purified from a human melanoma cell line contains a putative DPP4 truncation site,[46] while sequence from endogenous LIF purified from mouse Krebs II ascites carcinoma cells as reported does not have putative DPP4 truncation sites,[7] but

exogenous hLIF and mLIF produced in mouse Ehrlich ascites cells do have putative DPP4 truncation sites.[45] Thus, cellular context may be crucial to whether LIF is DPP4-truncatable or not. It is therefore critical to consider all possible isoforms when studying LIF, including the species that are DPP4-truncatable. In addition, when studying the effects of LIF protein in various cellular contexts by using rLIF, it is essential to know the N-terminal sequence of the recombinant protein that is produced and/or purchased. Many companies produce different versions of LIF that vary in their N-terminal sequence (Supplementary Table 3). We have demonstrated that these minor changes could affect their function in different cellular contexts. Thus, care should be taken to ensure that appropriate formulations of LIF are used for experiments where N-terminal PTM may play important roles in study outcomes.

A not-so-recent small phase I clinical trial showed that rhLIF might speed the recovery of hematopoiesis in patients receiving chemotherapy,[13] reiterating our hypothesis that LIF primarily functions in hematopoietic cells to regulate stressed hematopoiesis. While this trial showed some promise, the effect on neutrophil recovery was very small and effects on platelet recovery were modest, and follow-up studies do not seem to have been done. It is possible that the effects of hLIF treatment were muted by the presence of soluble or cell surface anchored DPP4, which we have demonstrated can truncate LIF and ablate its ability to preserve functional HPCs. Perhaps pre-treatment with an FDA-approved inhibitor of DPP-like Sitagliptin, which has been used to enhance granulocyte recovery after hematopoietic cell transplantation[56] and to lower incidence of graft-versus-host disease after allogeneic mobilized peripheral blood transplants,[57] may enhance recovery stimulating properties of hLIF.

Interestingly, both LIF and DPP4 have been implicated as playing a role in a variety of cancers, including both blood malignancies and solid tissue tumors. Breast cancer, pancreatic cancer, and bile duct cancers such as cholangiocarcinoma (to name a few examples) have had LIF implicated as a factor that promotes disease progression, growth, and/or survival.[32,50-52] Separately, DPP4 inhibition or dysregulation has been implicated to promote progression and growth in cancers of the same tissue subtypes.[53-55] It could be theorized that DPP4 inhibition could prevent truncation of LIF, which may, in turn, become a more potent driver of growth and survival in cancer cells. We postulate that DPP4 regulation of LIF may be an important and unstudied axis of regulation in these and other cancers, as well as in stressed hematopoietic contexts. This must be further explored and experimentally verified.

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Conflict of Interest

The authors declared no potential conflicts of interest in regard to this article.

Author Contributions

J.R.: Conception and design, collection of data, data analysis and interpretation, manuscript writing, final approval of manuscript. S.C.: Collection of data, final approval of manuscript. H.E.B.: Conception and design, financial support, collection of data, manuscript writing, final approval of manuscript.

Data Availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Supplementary Material

Supplementary material is available at *Stem Cells* online.

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