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A novel role for proliferin-2 in the ex vivo expansion of hematopoietic stem cells

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Abstract A family of proliferin genes was discovered on a microarray analysis of hematopoiesis supportive stromal cell lines. Proliferin-2 (PLF2) increased the frequency of long-term culture-initiating cells (LTC-IC) from 1 in 340 to 1 in 256 of the primary hematopoietic stem cell (HSC)-enriched bone marrow cells grown on MS5.1 feeder layer. A repeat using AFT024 feeder layer also showed a similar increase in LTC-IC (from 1 in 386 cells to 1 in 260 cells). The clonogenic output of the LTC-ICs was also increased significantly. The growth of various hematopoietic and stromal cell lines treated with PLF2 was found to increase by 4–27%, as measured by cell count and DNA synthesis assay. These findings open up the possibility of using PLF2 as a new member of the growth factor cocktails for the ex vivo expansion of HSC.

Key words: Hematopoietic progenitor cell; Stromal cell; Bone marrow; Fetal liver; In vitro expansion

1. Introduction

The use of stem cells for regenerative medicine and cell-based therapies is currently under intense exploration [1], and one of the best characterized of these are hematopoietic stem cells (HSC) [2]. HSC have important usage in a variety of clinical settings. They can be used as grafts to prevent chemotherapy-induced pancytopenia, as support to patients undergoing high-dose chemotherapy, as replacement gene therapy, and in immunotherapy by generating a large volume of immunologically active cells with anti-tumor activity [3]. The limitation to these applications has been in obtaining sufficient amounts of HSC or the total amount of defined target cells.

Adult bone marrow and fetal liver consist of both hematopoietic cells and the stroma needed to maintain hematopoiesis. HSC are closely associated with discrete in vivo stromal microenvironments. Recent efforts to understand the stromal microenvironments [4,5] have revealed that they involve a complex multi-component molecular network. The molecular signals that regulate and promote proliferation and other cell fates are produced by the cellular component of this microenvironment, which consists of fibroblasts, preadipocytes, endothelial cells and macrophages [6]. These microenvironments are thought to provide a rich milieu of molecular signals that mediate HSC differentiation and self-renewal through a combination of cytokine production, extracellular matrix component deposition, and cell–cell adhesion contacts with hematopoietic cells [6]. Identification and characterization of these regulators is an active area of research [4,5,7,8]. An understanding of the functions of these molecules will be the key to unlock the in vitro HSC expansion problem and to enhance bioengineering strategies to expand HSC in culture [3].

We used high-density microarrays in an effort to identify the various secreted and membrane molecules expressed by different stromal cell lines. A list of molecules associated with supportive activities was obtained by comparing supporting and non-supporting stromal cell lines. A family of glycoproteins known as the proliferins (PLFs) was discovered on the list. The PLFs comprise a group of four homologous proteins [PLF1, PLF2, PLF3, and PLF-related protein (PRP)] [9–13]. All forms of PLF show high similarity in their amino acid sequences. PLFs in adult mice has been shown to be expressed in the placenta, tail and ear skin, hair follicles, and small intestine [13–15]. Except for PRP, the presence of PLFs in these tissues is associated with growth (e.g. hair follicles and uterus), angiogenesis, and wound healing in the skin [13,15,16]. The homology between PLF and prolactin (31% sequence identity) puts them in the prolactin/somatotropin/placental lactogen family of peptide hormones. The growth-promoting effects of PLFs have not been demonstrated in adult HSC.

The above findings have led to the hypothesis that PLFs may be an autocrine or paracrine growth factor that regulates growth and differentiation of specific cells [16]. With this hypothesis in mind, we looked for PLF2 binding in hematopoietic progenitor cells and stromal cells, effects on the ex vivo expansion of primary adult HSC, and subsequently mitogenic activities in various cell lines.

2. Materials and methods

2.1. Cell lines

Mouse hematopoietic progenitor cell lines used in the study were EML and FDCP.Mix. EML is a bone marrow-derived progenitor cell line capable of differentiation along both lymphoid and myelo-erythroid pathways [17], while FDCP.Mix is another bone marrow-derived multipotential progenitor cell line [18]. For comparison purposes, M1 cells were used as a mature hematopoietic cell type capable only of differentiation along a single pathway [19]. The cell lines MS5.1 [20] and S17 [21] are bone marrow-derived stromal cells capable of maintaining primitive hematopoietic cells. OP9 [22] are stromal cells derived from mouse newborn calvaria. Two fetal liver-derived stromal cell lines used in this study were AFT024 and BFC012

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[7]. The maintenance and culture conditions of the cells were as described.

2.2. Isolation of primary bone marrow cells and enrichment for HSC

Total bone marrow was obtained from the femora of 8–12-week-old C57BL/6J mice and suspended in IMDM with 2% fetal bovine serum (FBS). Animals were killed by cervical dislocation; the procedure was approved by the Animal Holding Unit of the local institution. Fractions enriched with HSC were obtained by negative selection from the total bone marrow using the StemSep system (StemCell Technologies). Briefly, single cell suspensions were obtained from total bone marrow by vigorous pipetting. Mature hematopoietic cells were immunomagnetically labeled using a cocktail of antibodies (CD5, CD45R, CD11b, Ter119, Gr-1 and 7-4) directed against lineage markers. The cells were then passed through a magnetic column where the labeled cells (lineage-positive, Lin^+) would be captured and HSC-enriched cells (lineage-negative, Lin^-) would be collected from the flow-through.

2.3. Expression array analysis

Total RNA was extracted from the stromal cells using the commercial reagent Trizol (Invitrogen). cRNA synthesis and hybridization onto Affymetrix murine GeneChip MG_U74A ver2 were done according to the manufacturer's instructions. Expression profiling for each cell line was performed twice and the average expression level analyzed by the Affymetrix Microarray Suite 5.0 software. The measured mRNA expression levels from each cell type were normalized by dividing the individual mRNA value by the average mRNA expression of the cell, and analyzed using the GeneSpring software (Silicon Genetics). This allowed the comparison of mRNA expression across the different stromal cells.

2.4. Reverse transcription and semi-quantitative polymerase chain reaction (PCR)

The mRNA expression of PLF2 was confirmed by reverse transcription and semi-quantitative PCR analysis. Total RNA (5 μg) from the cells was reverse transcribed into first-strand cDNA using oligo-dT primers (SuperScript First Strand Synthesis System, Invitrogen) according to the manufacturer's instruction. The first-strand cDNA products were stored at −20°C for subsequent studies. Primers for PLF2 (forward primer: 5′-ACAGCTAAGCCTGGGTAGGACTCT-3′; reverse primer: 5′-GATATTTCAGAAGCAGAGCACATGA-3′) were synthesized (Proligo). A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as control in the amplification. The primer sequences for GAPDH were 5′-ATGGTGAAGGTCGGCGTGAAC-3′ for the forward primer and 5′-TTACTCCTTGGAGGCCATGTAGGC-3′ for the reverse primer (Proligo). The amplified full-length products of both PLF2 and GAPDH spanned from the 5′-untranslated region to the 3′-untranslated region of the genes. The semi-quantitative cycling parameters were 94°C for 1 min, followed by 25–35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min. This was to determine the pre-saturation linear amplification cycles, and the midpoint of the linear amplification was chosen for semi-quantitative analysis.

2.5. Baculovirus expression system

The degree of PLF glycosylation may determine differences in the functional interactions and binding with PLF receptors in vivo [16]. PLF2 was hence expressed in a baculovirus system for this experiment. The full-length PLF2 gene was cloned and expressed in Hi5 insect cells using the pFastBac system (Invitrogen). Recombinant PLF2 from the culture supernatant was purified through the His tag using a commercial Ni-NTA column (Qiagen). Purified recombinant PLF2 was resolved on a 12% sodium dodecyl sulfate–polyacrylamide gel to analyze its purity and integrity. The concentration of purified protein was quantified using a colorimetry method (Coomassie Plus, Pierce).

2.6. Flow cytometry analysis

Cells were washed and resuspended in phosphate-buffered saline (PBS) with 2% bovine serum albumin (BSA) before incubation for 3 h at room temperature with 100 nM purified recombinant PLF2. After that, the cells were washed and blocked with 5% rabbit serum for 15 min at 4°C prior to staining with fluorescein isothiocyanate (FITC)-conjugated antibody against human IgG_1 (Fc) (Dako) for 15–20 min at 4°C. The cells were washed and resuspended in PBS with 2% BSA and 1 μg/ml propidium iodide. Stained cells were analyzed by FACSCalibur (Becton Dickinson) using the CellQuest software (Becton Dickinson). For primary hematopoietic cells, the phycoerythrin-conjugated antibody (Becton Dickinson) against stem cell antigen 1 (Sca1) was also used to identify the primitive hematopoietic cells from the HSC-enriched bone marrow extracts. All antibodies used were diluted to the manufacturers' specified concentrations prior to the flow cytometry experiments.

2.7. Assays for long-term culture-initiating cells (LTC-IC) and colony-forming cells (CFC)

The LTC-IC content of the HSC-enriched bone marrow cells was determined by a limiting dilution assay (LDA). Briefly, a feeder layer for LTC-IC from bone marrow was prepared using MS5.1 or AFT024 cells. The feeder layer was plated onto gelatinized 96-well plates at 3×10^4 cells/well. After the cells had attached overnight, mitomycin C (Sigma) at 0.75 μg/ml (optimal predetermined concentration) was added to the cells to prevent further cell growth [23]. Mitomycin C was washed away after 3 h of incubation with the cells. LDA was performed using murine myeloid long-term culture medium (MyeloCult, StemCell Technologies) supplemented with 1 μM hydrocortisone sodium hemisuccinate (StemCell Technologies). HSC-enriched bone marrow cells (isolated as stated above) were plated at varying cell numbers onto the mitomycin C-treated feeder layer with 25 replicates for each cell number. The cultures were maintained for 4 weeks at 33°C and 5% CO_2 with weekly half medium replacement. After 4 weeks, all the cells in the LTC were harvested by trypsinization and resuspended in IMDM with 2% FBS. The cells in each well were plated onto a 35-mm dish containing methylcellulose medium supplemented with transferrin, insulin, erythropoietin (EPO), interleukin (IL)-3, IL-6 and stem cell factor (SCF) (MethoCult, StemCell Technologies). The colony formation culture was incubated at 37°C and 5% CO_2. The proportion of negative wells (wells containing no detectable CFC) after 14 days was counted and analyzed to determine the frequencies of LTC-IC from each input of cells. Clonogenic output of the LTC-IC was determined by scoring the number of colonies (containing ≥ 30 cells each) in each positive well.

2.8. Cell proliferation and 5-bromo-2′-deoxyuridine (BrdU) incorporation assays

To determine cell proliferation, cells were seeded at 1×10^4 per well onto a 96-well plate. PLF2 was given to the cells at various concentrations and time intervals. The number of viable cells in each well was counted using a hemocytometer after staining the cells with trypan blue (Sigma). For the BrdU incorporation assay, cells were seeded at 5×10^3 per well onto a 96-well plate. These were incubated at 37°C for 48 h in the presence of 100 nM PLF2 prior to staining with 10 μM BrdU (Cell Proliferation ELISA, BrdU Kit, Roche) for 2 h. The cells were then washed, fixed, and the DNA denatured according to the manufacturer's instruction. The amount of incorporated BrdU was detected colorimetrically using peroxidase-conjugated anti-BrdU antibody. The absorbance of the samples was measured at 450 nm with reference wavelength at 690 nm. Both assays were carried out in five replicates, and the means and S.E.M. were calculated. Statistical significance was determined using Student's unpaired *t*-test.

2.9. Statistical analysis

All statistical analyses performed in this study (i.e. *t*-test, curve fitting, linear regression analysis, etc.) were carried out using the software Prism ver3.03 (Graphpad Software). LDA analyses were performed with the L-Calc software (Stem Cell Technologies) to determine the LTC-IC frequencies and 95% confidence intervals of fitted lines.

3. Results

3.1. Expression profiles of the PLF family

In this project, we employed a large-scale genomic approach to dissect the hematopoietic microenvironment in the hope of identifying regulators that mediate stem cell fates. In general, the supporting cell types AFT024 [7], MS5.1 [20] and S17 [21] had higher expressions of the PLFs compared to the

Table 1
mRNA expression profiles of the PLF family in stromal and hematopoietic cell lines

GenBank No.	Proliferin	AFT024		BFC012		MS5.1		OP9		S17	
		Normalized	Flags	Normalized	Flags	Normalized	Flags	Normalized	Flags	Normalized	Flags
A: Stromal cells											
K02245	Proliferin-1	0.27	P	0.02	A	1.75	P	0.02	A	1.55	P
K03235	Proliferin-2	1.07	P	0.03	A	7.68	P	0.01	A	16.84	P
X16009	Proliferin-3	0.66	P	0.08	A	7.92	P	0.10	A	14.94	P
K03237	PRP	0.01	A	0.01	A	0.01	A	0.01	A	0.01	P
B: Hematopoietic cells		M1		EML		FDCP.Mix					
		Normalized	Flags	Normalized	Flags	Normalized	Flags				
K02245	Proliferin-1	0.01	A	0.01	A	0.02	A				
K03235	Proliferin-2	0.10	A	0.01	A	0.01	A				
X16009	Proliferin-3	0.07	A	0.10	A	0.13	A				
K03237	PRP	0.01	A	0.01	A	0.01	A				

The expression levels were normalized against the total mRNA expression of each cell type. Absence (A) or presence (P) calls for a gene were determined by the Affymetrix Microarray Suite software.

non-supporting cell types BFC012 [7] and OP9 (Table 1). None of the hematopoietic cell types expressed any PLF. PLF2 had the highest mRNA expression among the PLF family members. Thus, it was chosen for further characterization of its effects in the hematopoietic system.

Expression profiles of PLF2 from the microarray analysis were confirmed by reverse transcription and semi-quantitative PCR. This method was chosen over the conventional Northern blot method as it permits detection of mRNA of all abundance levels and is much faster than hybridization analyses. Due to the low starting amount of single-stranded cDNA, a trial PCR experiment was performed to 24–34 cycles with two-cycle intervals. An aliquot of the PCR mixture at each interval was electrophoresed on a 2% agarose gel. Exponential phase of the PCR was determined visually and found to range from 26 to 30 cycles. Saturation was found to begin after cycle 32. We chose to stop the PCR at cycle 28 for the semi-quantitative analysis of PLF2 cDNA level in various cell lines (Fig. 1). At this non-saturated cycle, the amplified products of PLF2 were found to correlate well with the microarray analysis results in Table 1, thereby confirming the expression levels of PLF2. Non-saturating PCR on normalized starting single-stranded cDNA should give an accurate comparison of relative mRNA copy numbers [24,25].

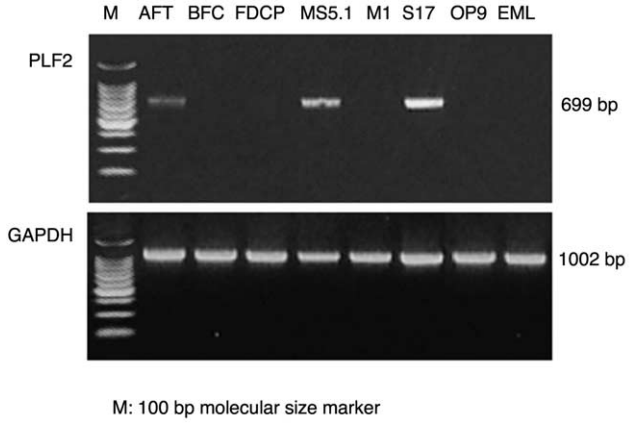


Fig. 1. Semi-quantitative PCR analysis of PLF2 gene expression in the stromal and hematopoietic cell lines. Equal amounts of first-strand cDNA product were used in the analysis. The GAPDH gene was used as a housekeeping gene to show equal amplification in all cell types. The images show the amplification products on a 1% agarose gel after 28 cycles.

3.2. PLF2 binding on hematopoietic and stromal cells

Purified recombinant PLF2 was tested for receptor binding in the stromal and hematopoietic cells. PLF2 was found to have greater binding to the EML hematopoietic progenitor cells than to the mature M1 hematopoietic cells (Fig. 2A). The bone marrow stromal cell line MS5.1 was found to have a higher intensity of fluorescence resulting from binding of FITC-labeled PLF2, suggesting the presence of more PLF2 receptors on these cells as compared to the fetal liver stromal cell line AFT024. PLF2 binding to primary bone marrow hematopoietic cells was also tested, where it seemed to bind specifically to the HSC-enriched (Lin^-) fraction of the bone marrow cells, but not to the Lin^+ cells (Fig. 2B). The findings that PLF2 could bind to the stromal cells and HSC-enriched Lin^- cells, but not to mature cells (M1 and Lin^+ cells) indicate that PLF2 may play a role in early hematopoiesis even though the specific PLF2 receptor in the hematopoietic system has not been identified.

Sca1 is a surface protein expressed on immature hematopoietic progenitor cells [26]. PLF2 identified a subpopulation of Sca1-positive primary bone marrow cells and also a group of cells negative for Sca1 (Fig. 2C). It is possible that PLF2 is identifying a subset of multi-lineage HSC that is receptive to long-term culture which may or may not be Sca1-positive. A group of cells known as the 'side population' cells have previously been identified to have high repopulating activity and multi-lineage potential despite the fact that only 75% of these cells are Sca1-positive [27]. It is unclear at this stage whether PLF2-positive cells are also within the 'side population' of HSC.

3.3. Effects of PLF2 on the frequency of LTC-IC and clonogenicity in HSC-enriched bone marrow cells

LDA was used to estimate the frequencies of LTC-IC in the HSC-enriched bone marrow cells [28]. In this study, LDA was performed on MS5.1 or AFT024 feeder layers. A linear relationship was obtained when the logarithm of the proportion of negative cultures was plotted against the total number of cells used to initiate each culture. The generalized linear model was fitted by finding the maximum likelihood using the Newton–Raphson method, and the goodness-of-fit to the model

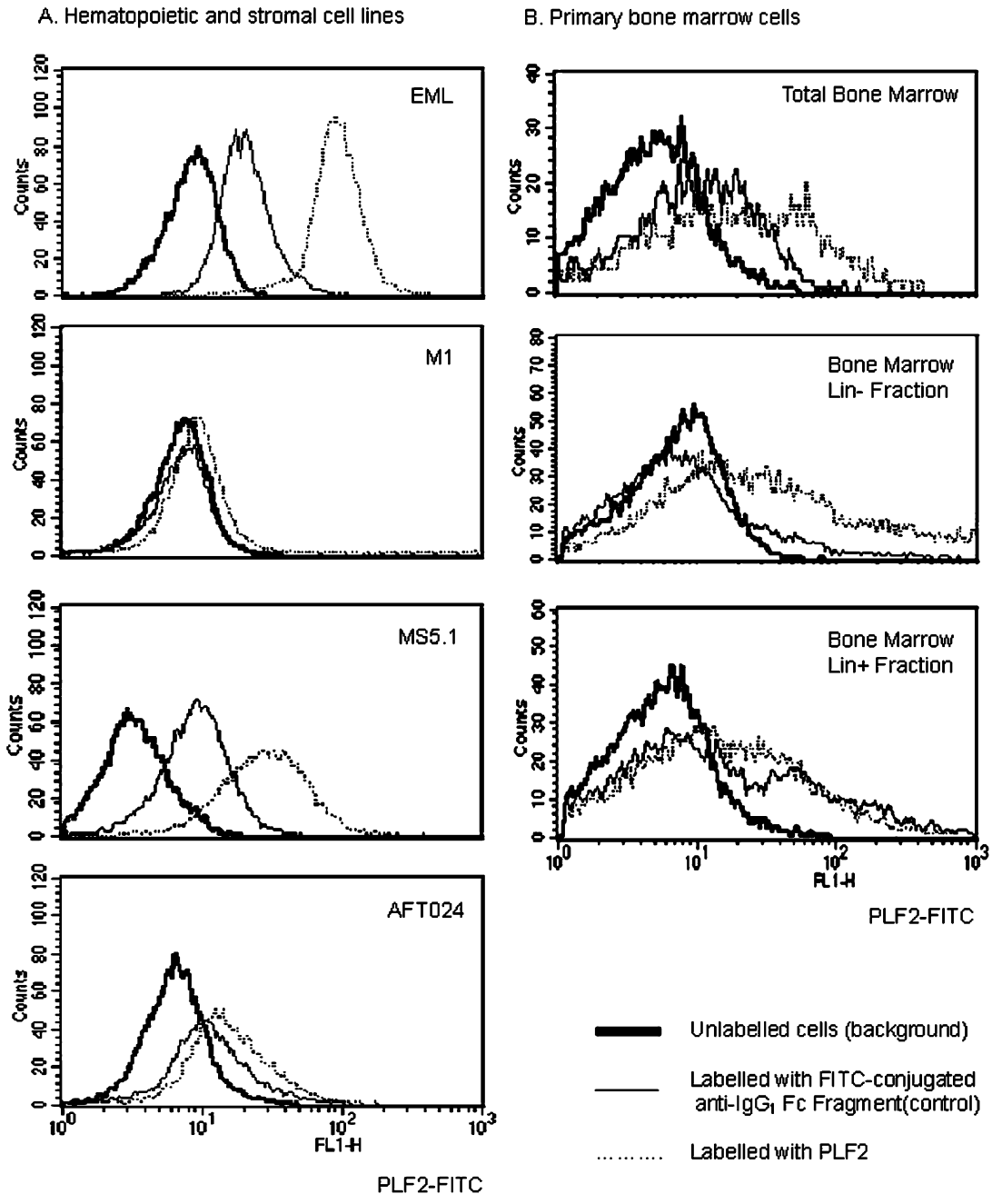


Fig. 2. Receptor binding of PLF2 on the stromal and hematopoietic cells. Binding of PLF2 to cells was detected by FITC-labeled secondary antibody using the FL-1 channel and is displayed as frequency histograms (A,B). Dot plots of Sca1 (FL-2 channel) and PLF2 (FL-1 channel) binding in primary bone marrow cells are also shown (C).

was assessed by the generalized Pearson chi-square. The LTC-IC frequency in the HSC-enriched bone marrow cells would then correspond to the derived cell dose at which 37% of the cultures yielded a negative response. Using MS5.1 as the feeder layer (Fig. 3A), the frequency of LTC-IC was 1 per 340 in C57BL/6 mouse-derived HSC-enriched bone marrow cells. A 1.3-fold ($P = 0.235$) increase in the frequency of LTC-IC (from 1 in 340 to 1 in 256 cells) was observed when 100 nM PLF2 was added to the medium. A similar experiment repeated with AFT024 as the feeder layer (Fig. 3B) showed an increase of 1.5-fold ($P = 0.06$) in LTC-IC (from 1 in 386 to 1 in 260 cells).

The number of clones (≥ 30 cells) in the positive CFC wells in each dilution was also counted and plotted against the initial cell seeding in LTC (Fig. 4). For the experiment using AFT024 as the feeder layer, the test for the null hypothesis that the two fitted lines were parallel was rejected ($P < 0.001$ by analysis of covariance). This showed that the increase in clonogenic output after PLF2 treatment was in direct proportion to the increase in LTC-IC frequency. The slopes of the two fitted lines in the experiment using MS5.1 as the feeder layer were not significantly different from each other ($P = 0.609$). A second null hypothesis that the lines were identical was tested. The two fitted lines were found to be distinct

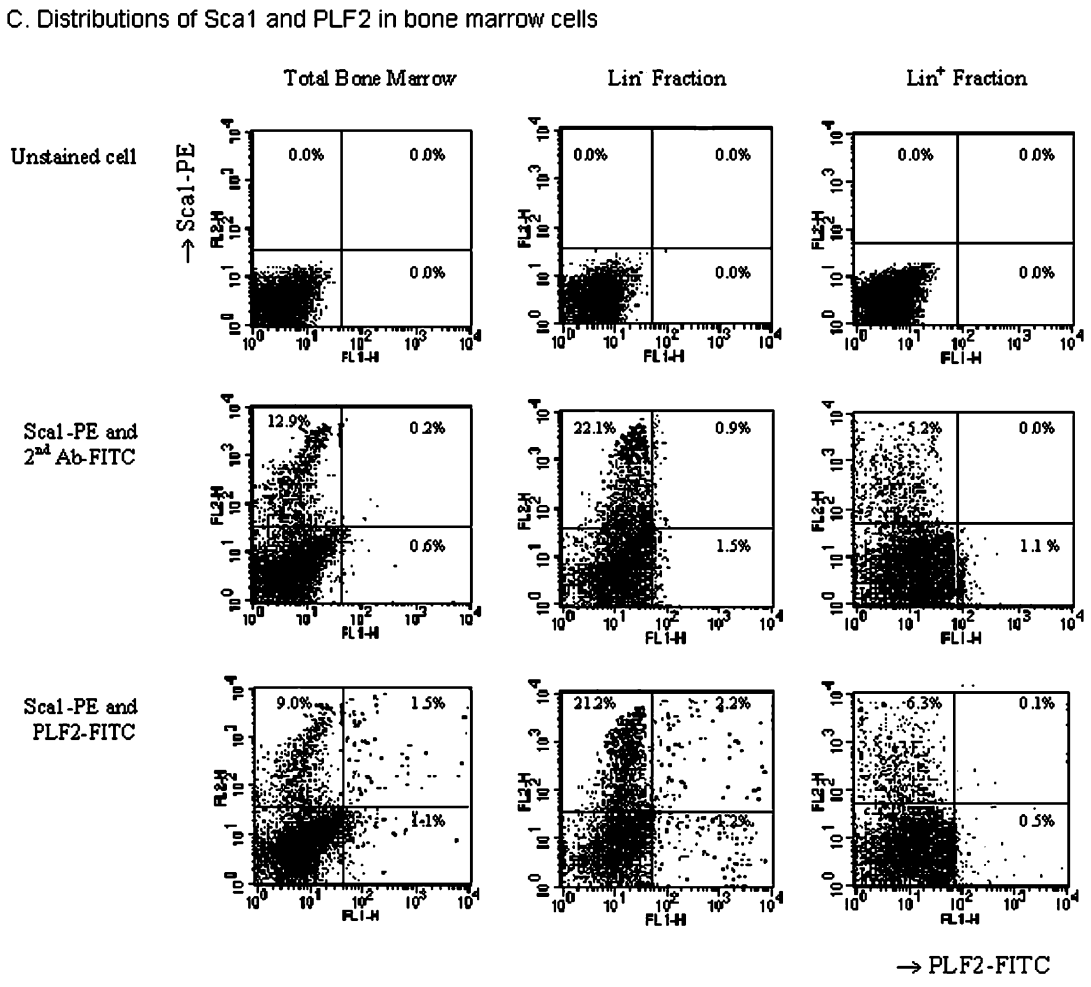


Fig. 2 (*Continued*).

but parallel to each other ($P = 0.004$), suggesting that the effect of PLF2 in increasing clonogenicity of LTC-IC cultured with the MS5.1 feeder layer was significant but low. These findings demonstrate that different stromal cells had different supporting activity on the long-term culture of HSC, with AFT024 supporting a higher number of clonogenic cells as compared to MS5.1 stromal cells.

Progenies that differentiated along different lineages were noted when the colonies were counted. The type of lineage observed was consistent with the type of growth factor (EPO, IL-3, IL-6 and SCF) incorporated into the methylcellulose medium. The colonies were almost exclusively of the multilineage potential type, i.e. colony-forming unit granulocyte-macrophage, and CFU-granulocyte, erythroid, macrophage, megakaryocyte, showing that LTC-ICs expanded by PLF2 still retained the capacity to differentiate along multiple lineages. An accurate quantitation of all types of progenies was not carried out due to difficulties in accurately assigning lineages to the clones.

3.4. Effects of PLF2 on hematopoietic and stromal cell lines

Next, we investigated the proliferation and pro-survival effects of PLF2 in the hematopoietic system. Initially, PLF2 was given daily or on alternate days to the cells, and the cell number was determined after 97 h. It was observed that PLF2 exerted an inhibitory effect on cell number (by 9–65%) on the various hematopoietic progenitor and stromal cell lines studied (data not shown). The successive administration probably caused a continual receptor stimulation resulting in down-regulation via internalization and degradation of the signal receptors. This made the cells refractory to additional PLF2. PLF2 was also given at various concentrations to the above cell lines. Concentrations lower than 100 nM did not seem to induce a significant increase in cell number or DNA synthesis as measured by BrdU incorporation assays (data not shown).

For subsequent experiments, PLF2 at 100 nM was administered at the commencement of the test and only during medium change after 96–120 h. There was an increase in bone marrow stromal MS5.1 cell number by 26.7% at 96 h after treatment with 100 nM PLF2 ($P = 0.116$) (Fig. 5). For the AFT024 fetal liver stromal cells, PLF2 at 100 nM stimulated an increase of 7.5% in cell number after 96 h ($P = 0.258$). PLF2 was also observed to have a slight proliferative effect on the EML hematopoietic progenitor cells (a 4.2% increase in cell number after 96 h, *P*0.416).

The effects of PLF2 on cells were further confirmed using the BrdU incorporation assay. The assay specifically measures active DNA synthesis during S phase of the cell cycle. The BrdU incorporation correlated well with the above cell count

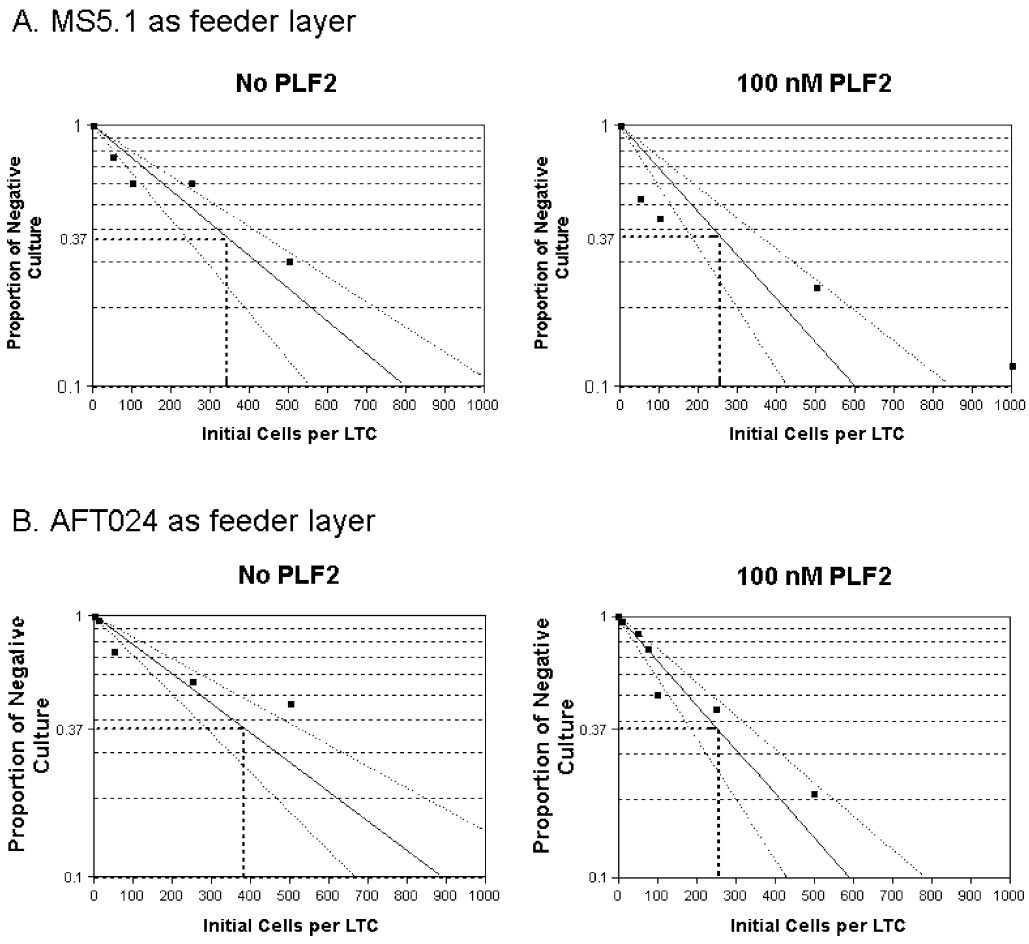


Fig. 3. Limiting dilution analysis of LTC-IC frequency in 100 nM PLF2-treated HSC-enriched bone marrow cells grown on MS5.1 (A) and AFT024 (B) stromal layers. The 95% confidence interval is shown as the dotted lines on either side of the fitted line. HSC-enriched cells grown on the MS5.1 feeder layer had a LTC-IC frequency of 1 in 340 cells (95% confidence interval: 1 in 244 to 1 in 473 cells), while those receiving PLF2 had a LTC-IC frequency of 1 in 256 cells (95% confidence interval: 1 in 185 to 1 in 355 cells). HSC-enriched cells grown on the AFT024 feeder layer had a LTC-IC frequency of 1 in 386 cells (95% confidence interval: 1 in 287 to 1 in 519 cells), while those receiving PLF2 had a LTC-IC frequency of 1 in 260 cells (95% confidence interval: 1 in 198 to 1 in 342 cells).

assay. MS5.1 stromal cells showed a significant 1.6-fold increase in DNA synthesis after treatment with PLF2 ($P = 0.014$), while the AFT024 stromal cells also showed a significant 1.7-fold increase in DNA synthesis ($P = 0.034$). From the above observations, PLF2 demonstrates an autocrine stimulatory effect on the stromal cells.

The EML hematopoietic progenitor cells treated with 100 nM PLF2 had higher BrdU incorporation (by 1.13-fold) as compared to untreated cells ($P = 0.479$). EML is a factor-dependent hematopoietic cell line (dependent on SCF). The cells will undergo cell death 24–48 h after withdrawal of the growth factors [17], shown here as a reduction in DNA synthesis. The present study shows that even though PLF2 can increase DNA synthesis in the hematopoietic progenitor cell line, it could not exert its effect without the presence of the required growth factor.

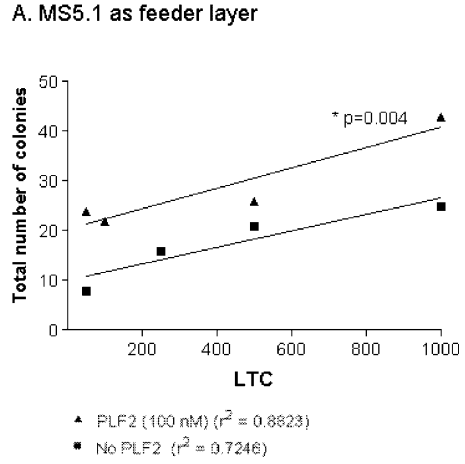
4. Discussion

PLFs show high amino acid sequence similarity among the members, and to the prolactin and placental lactogen. The PLFs' amino acid sequence conservation is high across the mammalian and reptilian kingdoms except for human, where there is no significant PLF homologue. On the other hand, domain homology search using Pfam revealed that it belongs to the somatotropin hormone family. The PLF2 amino acid sequence from 12 to 224 showed significant domain match to the human lactogen and somatotropin (Pfam score = 348.5, E value = 5.9e-102). The somatotropin hormone family consists of somatotropin, which plays an important role in growth control, choriomammotropin (lactogen), its placental analogue; prolactin, which promotes lactation in the mammary gland, and placental prolactin-related proteins [29]; proliferin and proliferin-related protein [12]; and somatolactin from various fishes [30]. PLF has been shown to bind to the insulin-like growth factor II (IGF-II)/mannose-6-phosphate (M6P) receptor in endothelial cells [31]. PLF can also bind with high affinity and specificity to an unknown specific receptor in the uterus that is distinct from the IGF-II/M6P receptor, and is able to mediate cell proliferation [32]. This unknown receptor may also be responsible for PLF transport though the yolk sac, which appears to be independent of the IGF-II/M6P receptor [33]. In all instances, binding of PLF is associated with cell growth and proliferation.

The dissociation constant, K_d, for PLF binding in uterine tissue [32] and fetal liver [34] is low (K_d value ranging from 0.6

to 2.0 nM). This suggests that PLF is a potent growth factor that can exert an effect at pico- to nanomolar concentrations. The maximal binding of PLF is observed in the femtomolar range per milligram uterine tissue-derived membrane protein [32], indicating that the numbers of PLF receptor are low per cell. This correlates well with our observation from flow cytometry analysis that the PLF2 FITC intensity shift profile is low for most cell types tested.

LTC-ICs are closely related to, if not highly overlapping with, transplantable stem cells. They are distinguished by their ability to generate multi-lineage colonies for at least 5 weeks in liquid cultures containing a stromal layer [35]. The in vivo competitive repopulation unit (CRU) assay tests the ability of a cell to generate and sustain production of both mature myeloid and lymphoid cells for many weeks after being transplanted into a hematologically compromised host. The assay has been adopted as an operationally useful, albeit cumbersome, definition for assigning a cell to the stem cell compartment. Until a simpler assay is developed, the test for the frequency of LTC-IC remains a useful alternative. Both the in vitro LTC-IC and the in vivo CRU assays are known to identify closely related populations in normal murine and human hematopoietic tissues of either adult [35] or fetal [36] origin.



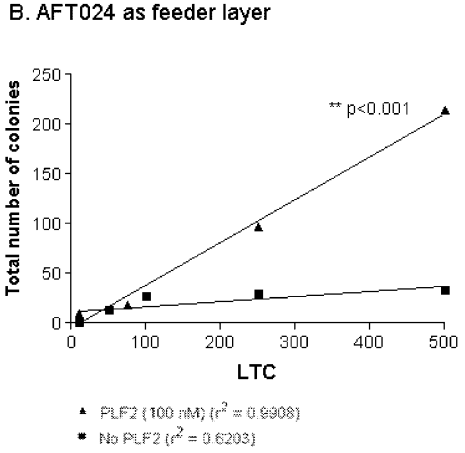


Fig. 4. Clonogenicity of LTC-IC administered 100 nM PLF2 grown on MS5.1 (A) and AFT024 (B) feeder layers. LTC-IC grown on the MS5.1 feeder layer showed a distinct and significant increase ($P = 0.004$) in clonogenicity when given PLF2. Similarly, LTC-IC grown on the AFT024 feeder layer showed a very significant increase ($P < 0.001$) in clonogenicity when given PLF2. From the slopes of the data sets, the increase in clonogenicity with AFT024 as the feeder layer was more drastic compared to using MS5.1 as the feeder layer.

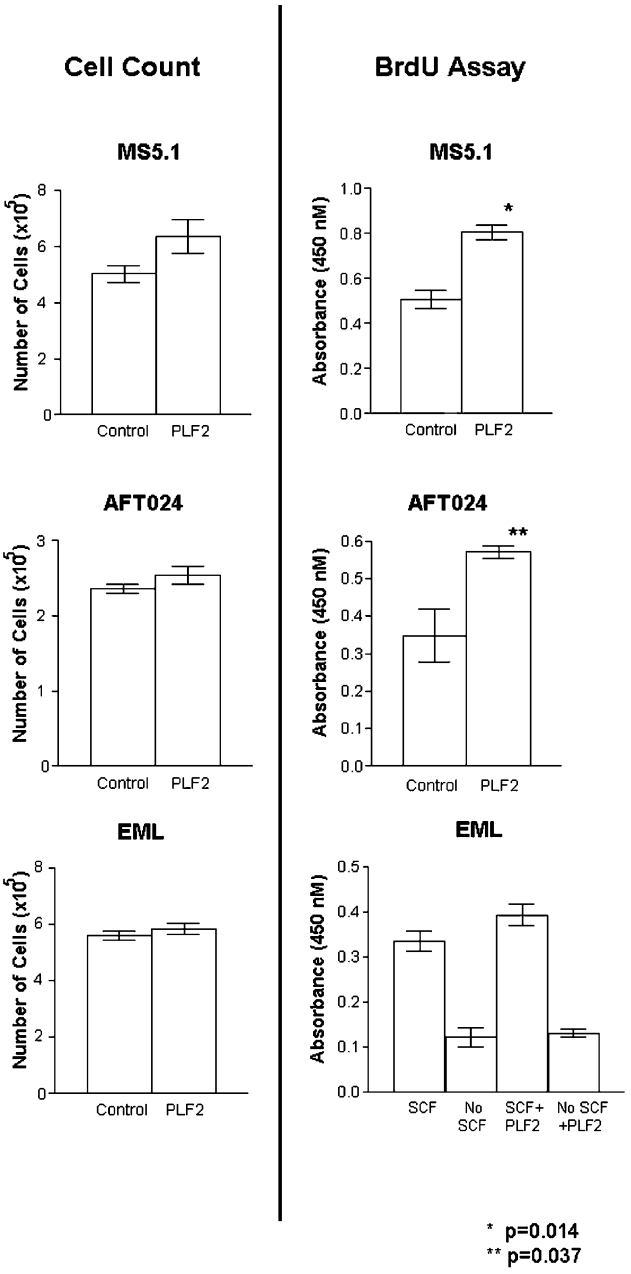


Fig. 5. Effects of PLF2 on cell proliferation. Viable cell count (A) and BrdU assays (B) were performed 96 h after administration of 100 nM PLF2. The controls were cells without PLF2 treatment. Results are shown as means with S.E.M. indicated by the error bars.

The moderate effect of PLF2 is probably not unexpected as HSC are exposed in situ to many different growth factors. Our current knowledge of how different soluble growth factors control the fate of HSC is deduced from in vitro studies, where the growth factors may not necessarily be introduced at physiological concentrations, in the correct ratios, or at the appropriate times. Some of the growth factors that have been shown to enhance the proliferation of HSC are flt3, SCF,

EPO, IL-6 and thrombopoietin [28,37]. Their effects are dependent on both the concentration of each component and the specific combinations of these growth factors. The combination of SCF, IL-3, IL-6 and soluble IL-6 receptor [38,39] is among the well-known cocktails for ex vivo expansion of human HSC. Thus, the use of a single growth factor may not allow full demonstration of its capacity. Further effort to delineate and optimize the activity of PLF2, alone or in conjunction with pre-existing growth factor cocktails, for the in vitro expansion of HSC is therefore warranted.

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