

Lineage-Restricted Expression of Bone Morphogenetic Protein Genes in Human Hematopoietic Cell Lines

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ABSTRACT: To explore the possibility that bone morphogenetic proteins (BMPs) are autocrine/paracrine regulators of hematopoietic differentiation and function, we screened a panel of human cell lines encompassing the hematopoietic lineages for expression of members of this family of genes. Expression of *BMP-2*, *BMP-4*, *BMP-6*, *BMP-7*, *Growth and Differentiation Factor-1 (GDF-1)*, *Placental Bone Morphogenetic Protein (PLAB)*, and *Transforming Growth Factor- β 3 (TGF- β 3)* was detected in one or more cell lines. *BMP-2*, *BMP-4*, *BMP-7*, and *TGF- β 3* expression was also found in normal hematopoietic tissue. Expression of *BMP-5* and *BMP-8* was not seen. Lineage-restricted patterns of expression were found for *BMP-4* (T-lymphoid), *BMP-7* (lymphoid), *PLAB* (macrophage/monocyte), and *GDF-1* (myeloid). Expression of *BMP-2*, *GDF-1*, and *PLAB* could be modulated by treatment with differentiating agents. Marked variations in the levels of *BMP-4*, *BMP-7*, and *PLAB* expression were encountered, indicating that disorders in BMP signaling pathways may play a role in the development of hematopoietic neoplasia.

Keywords: bone morphogenetic proteins, lineage-restricted expression, hematopoiesis, differentiation, hematopoietic cell lines, neoplasia

INTRODUCTION

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-beta (TGF- β) superfamily of signaling polypeptides. These cytokines regulate cell proliferation, differentiation, morphogenesis and apoptosis. During embryogenesis BMPs are involved in the establishment of pattern formation and in the development of tissues and organs; in mature tissues these peptides maintain tissue homeostasis (1, 2). The genes that encode bone morphogenetic proteins are part of developmental regulatory circuits both upstream and downstream of homeobox-containing genes (3-5). The homeobox genes are a family of developmentally significant transcription factors which, in addition to determining cell fate during embryogenesis (6), are implicated in the control of lineage specificity in hematopoiesis (7-9). A prominent theme in modern developmental biology is that similar regulatory pathways are involved in multiple systems. Accordingly, the bone morphogenetic proteins are plausible

candidates for regulators both of hematopoietic differentiation and of function in mature blood cells.

Recent studies point to a function or functions for the bone morphogenetic proteins in hematopoiesis. Bone morphogenetic protein 4 (BMP-4) induces formation of embryonic hematopoietic tissue (10, 11). Subcutaneous implantation of BMP-2 is capable of inducing a hematopoietic microenvironment that supports clonogenic lymphoid and myeloid progenitors (12). Other members of the TGF- β superfamily play multiple roles in hematopoiesis. Activin A promotes erythroid differentiation by stimulating the proliferation and differentiation of interleukin-3 (IL-3)-responsive erythroid burst-forming units while inhibiting the proliferation of IL-3-responsive granulocyte-monocyte colony forming units (13). Activin A is also produced by peripheral blood monocytes activated with lipopolysaccharide or 1,25-dihydroxyvitamin D3 (14, 15). The isoforms of TGF- β exert both stimulatory and inhibitory effects on hematopoietic progenitor cells (16-18).

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Expression of the genes for the BMP type I receptors and their downstream signal transducers has been found in hematopoietic stem cells (19). Recently BMP-9 has been shown to act as a hematopoietic hormone (20).

The regulation of hematopoiesis requires inductive signaling among stromal cells, stem cells, and progenitor cells. Such complex signaling may involve concentration gradients of morphogens, competition between morphogens, and differential regulation by heterodimer and homodimer formation. All these mechanisms have been proposed for the BMPs. This study examines the possibility that BMP genes are expressed by developing hematopoietic cells, presumably as part of an autocrine/paracrine mechanism regulating development. Our expectation was that we would find expression of bone morphogenetic protein genes in hematopoietic cell lines, that expression patterns would correlate with hematopoietic lineage, and that the amount of message detected would be modulated by treatment of the cells with differentiating agents. This is the first report of the expression of these genes in hematopoietic cell lines and in normal adult (as opposed to embryonic) hematopoietic tissue. We found expression of *BMP-2*, *BMP-4*, *BMP-6*, *BMP-7*, *Placental Bone Morphogenetic Protein (PLAB)*, *Growth and Differentiation Factor-1 (GDF-1)*, and *Transforming Growth Factor- β 3 (TGF- β 3)*. Expression of *BMP-5* and *BMP-8* was not found. Lineage-restricted patterns of expression were found. Expression of *BMP-4* is found only in T-lymphoid cell lines. *BMP-7* expression is found in normal circulating lymphocytes and in Ramos, a B-cell line. *PLAB* expression is found in cell lines with the capacity to differentiate along the monocyte/macrophage pathway, and *GDF-1* expression is predominantly myeloid. Expression of *BMP-2*, *GDF-1*, and *PLAB* is affected by differentiation. Marked elevation occurs in the production of *BMP-4*, *BMP-7*, and *PLAB* message in some leukemia cell lines compared with normal tissue, suggesting that disorders of

BMP signaling are a factor in the development of hematopoietic malignancy.

MATERIALS AND METHODS

Cell Culture

Human leukemia cell lines were obtained from the American Type Culture Collection and maintained in RPMI-1640 medium containing 10% fetal bovine serum (Gibco BRL) in a 5% CO₂ incubator at 37 C. Granulocyte-monocyte colony stimulating factor (2 ng/ml) or interleukin-3 was added to cultures of TF-1 cells (Invitrogen). HL-60 cells were grown in medium containing 20% fetal bovine serum.

Macrophage differentiation was induced in Reh and TF1 cells by addition of 5 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma), in U937 cells by addition of 160 nM TPA, and in K562 cells by addition of 100 nM TPA. Induction of macrophage differentiation was monitored by the development of adherence. Adherent cells and cells remaining in suspension were harvested for RNA extraction after 24 hours for Reh and TF1, and after 48 hours for TF1. K562 cells were harvested after 24, 48, or 72 hours incubation with TPA.

Erythroid differentiation was induced in K562 cells by incubation with 50 μ M hemin (Sigma) prepared as described (21) for 24 or 48 hours before harvest and RNA isolation. Erythroid differentiation was induced in TF1 cells by incubation with 0.5 mM δ -aminolevulinic acid (ALA) for 72 hours.

Separation of Peripheral Blood Leukocytes and Bone Marrow Harvest

Human peripheral blood was drawn by standard venipuncture from normal volunteers after informed consent was obtained in accordance with protocols approved by the Institutional Review Board of the Medical Center of Central Georgia. Mononuclear cells were

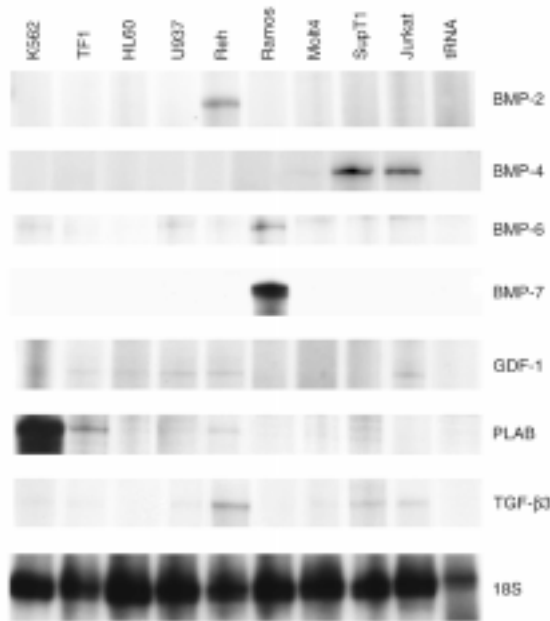


Figure 1. Expression of TGF- β -superfamily genes in human hematopoietic cell lines. Expression was determined by the RNase protection assay on 50 μ g samples of total RNA from the indicated cell lines using probes for TGF- β -superfamily genes. Protected bands were visualized by autoradiography or by Phosphorimager analysis. A probe for 18 S RNA was included in each experiment as a control for loading.

prepared from heparinized human peripheral blood by diluting blood 1:2 in phosphate-buffered saline and layering 40 ml of diluted blood over 10 ml Histopaque (Sigma; density 1.077 g/l) in 50 ml conical tubes (Fisher Scientific), followed by centrifugation at 800 x g for 30 minutes at room temperature. The buffy coat was resuspended in RPMI-1640 medium (containing 10% fetal calf serum; "complete media"). Adherent cells were removed by incubation of the cell preparation on 100 x 20 mm plastic plates (Fisher Scientific) that had been preincubated with 15 ml of fetal calf serum for 1 hour. After incubation on the plastic plates for 1 hour at 37 C, nonadherent cells (containing primarily T cells, B cells and natural killer cells) were decanted. The adherent cells (primarily monocytes) were harvested from the plastic plates by gentle scraping with a sterile plastic cell scraper and suspended in complete medium for later use. The nonadherent cells were layered onto nylon wool-packed columns that had been

presoaked with complete medium and incubated for 1 hour at 37 C to remove B cells. After incubation, the nylon-nonadherent cells (primarily T cells and natural killer cells) were flushed off the column using three washes of complete media (15 ml/wash). B cells were removed from the nylon wool by gentle compaction followed by flushing with complete media. Cell viability, as judged by trypan blue exclusion, was typically greater than 95% for all cell types.

Human bone marrow was aspirated from the iliac crest from normal volunteers after informed consent was obtained in accordance with protocols approved by the Institutional Review Board of the Medical Center of Central Georgia.

RNA Isolation

Total RNA was prepared from cells using the guanidine thiocyanate/acid phenol method (22) or by silica gel chromatography using an RNeasy kit (Qiagen). After quantitation by ultraviolet absorbance, the RNA was stored as a suspension in 0.3M sodium acetate/70% ethanol at -70 C.

Generation of BMP Probes

I.M.A.G.E Consortium (LLNL) cDNA clones (23) (Research Genetics) were obtained for *BMP-2*, clone ID: 754523; *BMP-4*, clone ID: 797048; *BMP-5*, clone ID: 1032405; *BMP-6*, clone ID: 768168; *BMP-7*, clone ID: 712899; *BMP-8*, clone ID: 926366; *GDF-1*, clone ID: 174239; *PLAB*, clone ID: 788832; and *TGF- β 3*, clone ID: 796607. Fragments of the EST clones were subcloned into the Bluescript II SK vector (Stratagene) and used to generate probes of suitable length to give a 200-400 nucleotide protected fragment in the RNase protection assay. A 365 base pair (bp) Xho I/Hinc II fragment of *BMP-2*, a 200 bp Pst I/Sma I fragment of *BMP-4*, a 304 bp Bst YI fragment of *BMP-5*, a 476 Pst I/Pml I fragment of *BMP-6*, a 393 bp BamH I/Msc I fragment of *BMP-7*, a 243 bp Xho I/Hinc II fragment of *GDF-1*, a 418 bp Apa I/Pvu II

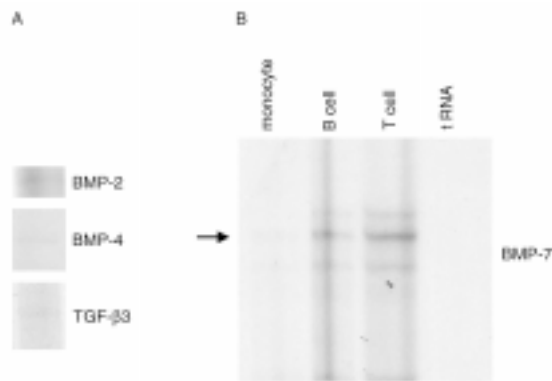


Figure 2. Expression of TGF- β -superfamily genes in normal hematopoietic tissue. Expression was measured by the RNase protection assay on 50 μ g samples of total RNA. A. Expression of *BMP-2*, *BMP-4*, and *TGF- β 3* in normal human marrow. B. Expression of *BMP-7* in fractionated circulating mononuclear cells.

fragment of *PLAB*, and a 400 bp Xho I/Sma I fragment of *TGF- β 3* were cloned into the appropriate sites in Bluescript. The *BMP-8* EST clone was used without subcloning. Following linearization with the appropriate restriction endonuclease, the DNA templates were transcribed with T3 or T7 RNA polymerase in the presence of 800 Ci/mMol α -[32 P]-UTP.

RNase Protection Analysis

The probes generated by transcription of the linearized subclones were purified by electrophoresis through a 7 M urea/5% polyacrylamide gel. The full-length transcript was eluted from the gel into the Probe Elution Buffer provided in the RPA II kit (Ambion). Each sample contained 50 μ g RNA coprecipitated with approximately 5×10^4 dpm probe. A probe for 18 S RNA was added as an internal control for loading. The pellets were dissolved in hybridization buffer and incubated overnight at 50 C. RNase digestion and precipitation of protected fragments was as described in the manufacturer's instructions. The protected fragments were detected by electrophoresis through a 7 M urea/5% polyacrylamide gel followed by autoradiography

with an intensifying screen at -70 C. Multiple exposures of the gels were made to obtain bands in the linear response range of the film. Quantitative densitometry was performed using the NIH Image program. (This program, written by Wayne Rasband at the National Institutes of Health, is available at <http://rsb.info.nih.gov/nih-image/index.html>.) In some experiments, the protected bands were visualized using a Storm Phosphorimager (Molecular Dynamics) and quantitated using the accompanying ImageQuant software.

Multiple densitometric measurements were analyzed by the independent Student's t-test to determine statistical significance.

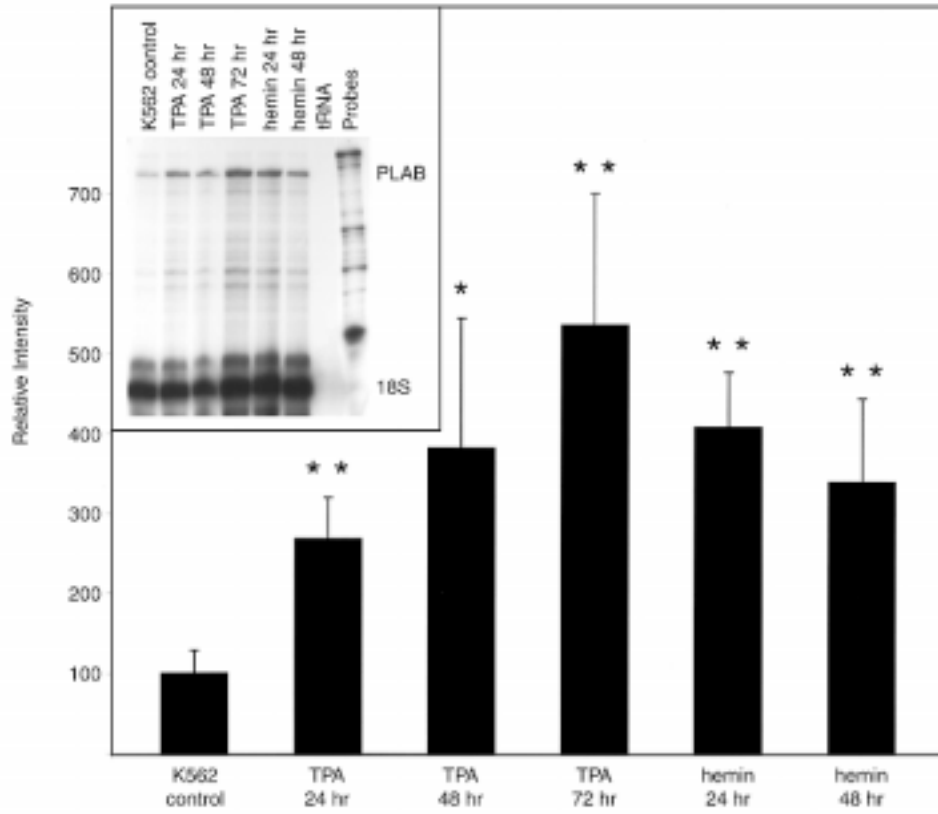
RESULTS

Expression Patterns of BMP Genes

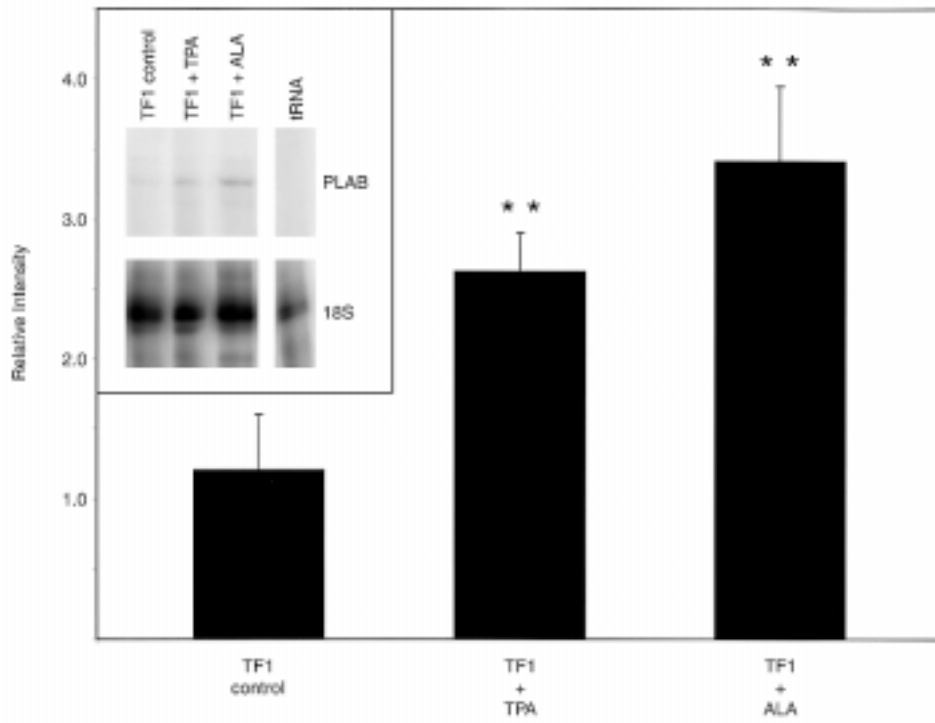
We used leukemia and lymphoma cell lines as a model of hematopoietic development. The cell lines retain features of the lineage and differentiation state from which they are derived. To ascertain whether bone morphogenetic proteins are potential autocrine/paracrine regulators of hematopoietic differentiation and function, we used the RNase protection assay to screen a panel of cell lines which encompass the hematopoietic lineages for expression of members of this family of genes. The following myeloid cell lines were selected: K562 and TF1, multipotent cell lines with erythroid features (24, 25); HL60, which consists predominantly of neutrophilic promyelocytes (26); and U937, a monocytic cell line (27). The following cell lines of lymphoid lineage were screened: Reh, a pre-B cell line (28); Ramos, an Epstein-Barr-negative Burkitt's lymphoma cell line (29); and the T-cell lines Molt4, SupT1, and Jurkat (30-32). In some cases, we also screened peripheral blood monocytes, B cells, T cells, and normal bone marrow.

Expression of *BMP-2* was found in Reh (Fig. 1) and in normal marrow (Fig. 2A). *BMP-4*

a



b



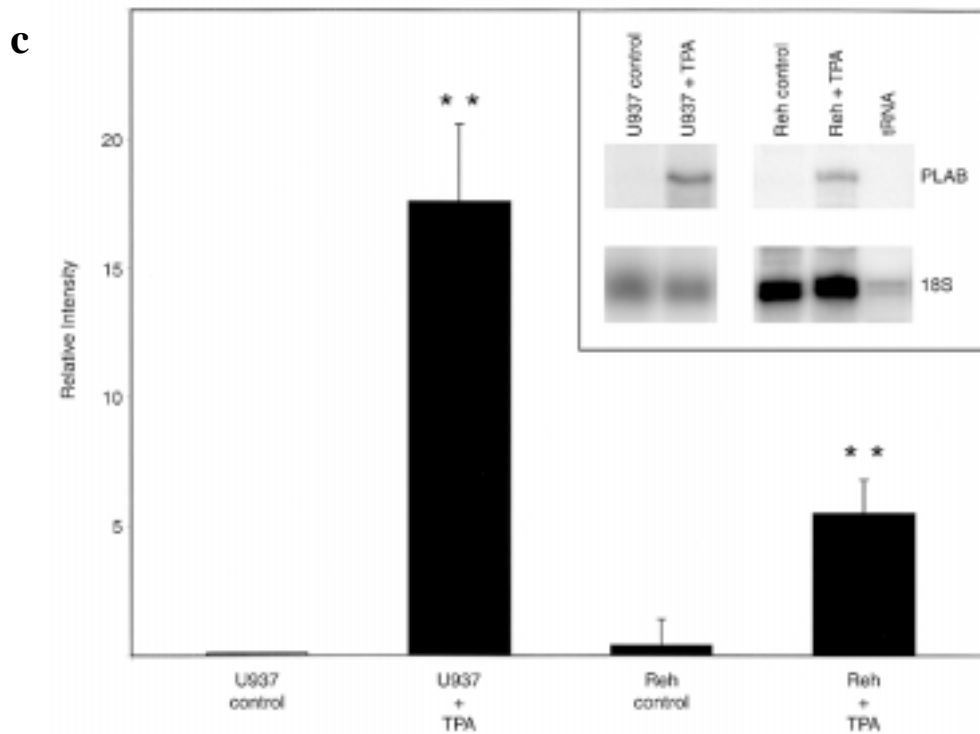


Figure 3. Effect of treatment with the differentiating agents on *PLAB* expression in hematopoietic cell lines. Expression was measured by the RNase protection assay on 50 μ g samples of total RNA. A probe for 18 S RNA was included as a control for loading. A. *PLAB* expression in K562 cells. Inset: Autoradiogram of a typical experiment. Lane 1, untreated K562 cells; Lanes 2-4 after a 24, 48, or 72 hour incubation with 100 nM TPA; Lanes 5-6 after a 24 or 48 hour incubation with 50 μ M hemin; Lane 7, yeast tRNA; Lane 8, probes. Bar Graph. Densitometric analysis of *PLAB* expression following treatment with differentiating agents. Densitometry of the *PLAB* and 18 S protected bands were analyzed using the NIH image program. The *PLAB* intensities obtained were corrected to a constant 18 S intensity, and the control value was set at 100. Data represent the mean plus the standard deviation from 4 experiments. B. *PLAB* expression in TF-1 cells. Inset: Phosphorimager generated autoradiograms. Lane 1, untreated TF1 cells; Lane 2, after a 48 hour treatment with 5 nM TPA; Lane 3, after a 72 hour treatment with 0.5 mM δ -aminolevulinic acid (ALA); Lane 4, tRNA. Bar graph. Quantitative analysis of *PLAB* expression following treatment with differentiating agents. Data were collected with a Phosphorimager and analyzed with the ImageQuant program. The experimental bands were normalized to a constant 18 S value. Data represent the mean plus the standard deviation from at least 4 measurements. C. *PLAB* induction in U937 and Reh cells. Inset: Phosphorimager generated autoradiograms. Lane 1, untreated U937; Lane 2, U937 after treatment with 160 nM TPA for 24 hours; Lane 3, untreated Reh; Lane 4, Reh following treatment with 5 nM TPA for 24 hours; Lane 5, tRNA. Bar Graph. Quantitative analysis of *PLAB* expression following treatment with TPA. Data were analyzed as in B and represent the mean plus standard deviation from 2 measurements (U937) or 5 measurements (Reh). Confidence values were determined by the student's independent t-test: *, $p < 0.05$; **, $p < 0.01$.

expression was detected only in the T-cell lines Molt4, SupT1, and Jurkat, but not in the B-lymphoid cell lines, suggesting a role for *BMP-4* in T-cell function. The myeloid cell lines also did not express *BMP-4* within the limits of the sensitivity of the RNase protection assay; however, low levels of *BMP-4* expression could be detected in normal marrow.

BMP-2 and *BMP-4* expression in normal marrow is consistent with a role for these genes in the control of adult, in addition to embryonic, hematopoietic development. *BMP-2* and *BMP-4* are implicated in the induction of hematopoietic tissue that takes place as part of pattern formation in the mesoderm (10, 11, 33). The primary effect in the *Xenopus* embryonic system appears to be

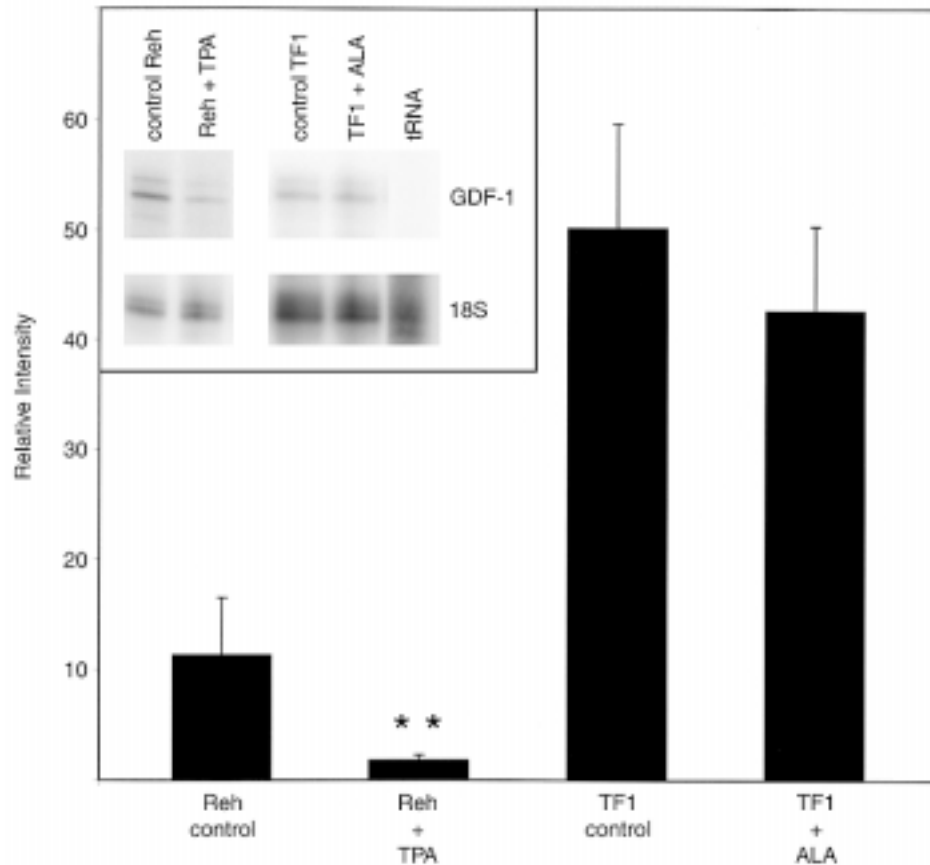


Figure 4. Effect of treatment with differentiating agents on *GDF-1* expression in hematopoietic cell lines. Expression was measured by the RNase protection assay on 50 μ g samples of total RNA. A probe for 18 S RNA was included as a control for loading. Inset: Phosphorimager generated autoradiogram of a typical experiment. Lane 1, untreated Reh cells; Lane 2, Reh cells treated with 5 nM TPA for 24 hours; Lane 3, untreated TF1 cells; Lane 4, TF-1 cells after treatment with 0.5 mM ALA for 72 hours; Lane 5, yeast tRNA. Bar Graph: Quantitative analysis of *GDF-1* expression. Data were collected with a Phosphorimager and analyzed with the ImageQuant program. The *GDF-1* bands were normalized to a constant 18 S value. Data represent the mean plus the standard deviation from 4 measurements. Confidence values were determined by the student's independent t-test: **, $p < 0.01$.

the induction of erythroid development. BMP-4 protein treatment of isolated hematopoietic stem cells affects proliferation, differentiation and length of stem cell survival (19). The presence of *BMP-4* message in the lymphoid lines thus suggests that this cytokine has multiple roles in the development and maintenance of the blood. We were unable to detect *BMP-4* expression in normal circulating monocytes, B cells, or T cells. However, *BMP-4* expression has been detected by the polymerase chain reaction in CD34+ cells, normal monocytes, and T cells, but not in B cells, (34) at least partly consistent with the results obtained from the screen of the cell lines.

BMP-6 and *BMP-7* were detected in Ramos (Fig. 1). Expression of these genes was not detected in the pre-B lymphoid cell line Reh, the T-lymphoid cell lines Molt4, SupT1, and Jurkat, or any of the myeloid cell lines and bone marrow. *BMP-7* message was not detected in peripheral blood monocytes, but was seen in preparations of circulating B cells and T cells (Fig. 2B). These results suggest a role for *BMP-7* in the development and/or function of cells of lymphoid lineage. It should be noted that the Expressed Sequence Tag clone obtained for *BMP-7* used to generate the probes in these experiments was derived from human tonsil cells enriched for

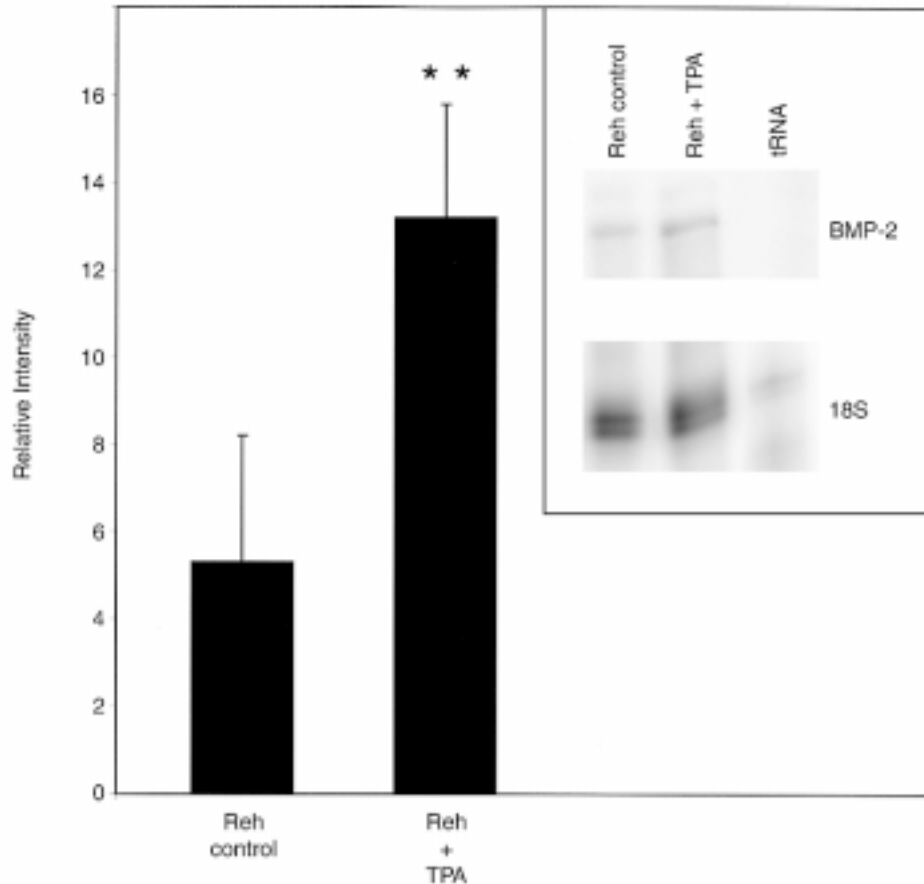


Figure 5. Effect of TPA on *BMP-2* expression in Reh cells. Expression was measured by the RNase protection assay on 50 μ g samples of total RNA. A probe for 18 S RNA was included as a control for loading. Inset: Phosphorimager generated autoradiogram of a typical experiment. Lane 1, untreated Reh cells; Lane 2, Reh cells treated with 5 nM TPA for 24 hours; Lane 3, yeast tRNA. Bar Graph: Quantitative analysis of *BMP-2* expression. Data were collected with a Phosphorimager and analyzed with the ImageQuant program. The *BMP-2* bands were normalized to a constant 18 S value. Data represent the mean plus the standard deviation from 4 measurements. Confidence values were determined by the student's independent t-test: **, $p < 0.01$.

germinal B cells (35), consistent with a lymphoid role for *BMP-7*.

TGF- β 3 and *GDF-1* message were found in multiple cell lines (Fig. 1), and in the case of *TGF- β 3*, in normal marrow as well (Fig. 2A). These genes were selected as a presumed positive and negative control, respectively. *TGF- β 3* transcripts were found in the K562, TF1, U937, Reh, Molt4, SupT1, and Jurkat cell lines; thus, *TGF- β 3* is produced by cells of both myeloid and lymphoid lineage. The *TGF- β 3* peptide affects both myeloid and lymphoid cells (17, 36). The functions of the *TGF- β* isoforms in hematopoietic tissue have been extensively

studied; the detection of *TGF- β 3* transcript in hematopoietic cell lines was not surprising. The presence of *GDF-1* transcripts in multiple cell lines was unexpected; *GDF-1* has been thought to be specific to neural tissue (37). However, it was found in all the myeloid cell lines except K562, the primitive lymphoid cell line Reh, and in the mature T-lymphoid line Jurkat. These results suggest that at minimum, *GDF-1* plays a role in early myeloid development and may have a function in lymphocytes.

RNase protection analysis of the panel of cell lines with a probe for *PLAB* shows that *PLAB* message is abundant in K562 cells compared with the amount of message in TF1

Table 1. Expression Patterns of Bone Morphogenetic Protein Genes

		BMP-2	BMP-4	BMP-5	BMP-6	BMP-7	BMP-8	GDF-1	PLAB	TGF-β3
cell line	lineage									
K562	erythroid	-	-	-	-	-	-	-	+,a	+
TF-1	erythroid	-	-,c	-	-	-	-	+,c	+,a	+
HL60	neutrophil	-	-	-	-	-	-	+	-	-
U937	monocyte	-	-,c	-	-	-	-	+	-,a	+
Reh	Pre B	+, a	-,c	-	-	-	-	+,b	+/-,a	+
Ramos	B cell	-	-	-	+	+	-	-	-	-
Molt4	T cell	-	+	-	-	-	-	-	-	+
Sup-T1	T cell	-	+	-	-	-	-	-	-	+
Jurkat	T cell	-	+	-	-	-	-	+	-	+
normal tissue										
bone marrow		+	+	-	-	-	-	-	-	+
monocytes		ND	-	ND	ND	-	ND	ND	-	ND
B cells		ND	-	ND	ND	+	ND	ND	-	ND
T cells		ND	-	ND	ND	+	ND	ND	-	ND

Note. ND, not determined; a, increases in response to differentiating agents; b, decreases in response to differentiating agents; c, does not change in response to differentiating agents.

and Reh cells (Fig. 1). Although these cell lines are members of different hematopoietic lineages, all three cell lines have the potential to differentiate along the macrophage pathway in response to suitable inducers. Induction of macrophage differentiation increases the amount of *PLAB* message present (discussed below). *PLAB* message has been detected in activated normal macrophages in vitro (38).

BMP-5 and *BMP-8* expression was not detected in any of the cell lines.

Modulation of BMP Message

One hypothesis underlying this study is that the individual bone morphogenetic proteins regulate hematopoietic development in a lineage-specific and differentiation-stage-specific manner. A test of the hypothesis is to determine if the amount of a BMP message varies in response to differentiating agents.

As *PLAB* expression appears correlated with the potential to differentiate along the macrophage lineage, we therefore have examined the effect of treatment of cell lines with differentiating agents on the amount of *PLAB* message detected. K562 and TF1 are both multipotent cell lines with erythroid features, and

macrophage differentiation can be induced in both by TPA. Erythroid differentiation can be induced by hemin and δ-aminolevulinic acid (ALA). TPA-induced macrophage induction of K562 cells (Fig. 3A) results in a statistically significant ($p < 0.01$) 3-5-fold increase in *PLAB* message. There is no statistically significant difference in the amounts of *PLAB* message produced at 24, 48, or 72 hours. Both the adherent and non-adherent fractions of TPA-treated K562 cells show a similar elevation of *PLAB* message. These results are consistent with *PLAB* expression being associated with macrophage differentiation. Unexpectedly, however, erythroid differentiation of K562 cells with hemin also results in a significant ($p < 0.01$) increase in *PLAB* message of 3-4 fold. Again, there is no significant difference between 24 and 48 hours in the amount of message produced in response to hemin.

Similar results were obtained when TF1 cells were treated with TPA or ALA (Fig. 3B) Macrophage induction with TPA resulted in a 2-fold ($p < 0.01$) increase in *PLAB* message. A 3-fold ($p < 0.01$) increase in *PLAB* message was detected following induction of erythroid differentiation with ALA.

Although the Reh cell line is considered a primitive lymphoid cell line, treatment with TPA induces macrophage differentiation (39). In the absence of TPA induction, only very low levels of *PLAB* message can be detected in some RNA preparations; following induction, *PLAB* message is readily seen (Fig. 3C). The increase in *PLAB* message is at least 10-fold ($p < 0.01$). Similarly, *PLAB* message is not detected in the monocytic cell line U937; however, induction of differentiation with TPA results in the activation of *PLAB*.

PLAB message is not detected in bone marrow, in preparations of peripheral monocytes, B cells, or T cells by the RNase protection assay (data not shown). However, *PLAB* expression has recently been reported in activated macrophages in culture and in the myelomonocytic cell line KG1 after activation with retinoic acid and TPA (38). These results, coupled with the results of the hematopoietic lineage and differentiation studies above, indicate that the *PLAB* gene is activated in cells with the potential to become macrophages. The observation that *PLAB* message increases upon the induction of erythroid differentiation in two cell lines raises the possibility that the *PLAB* gene also function in early erythroid differentiation. Another possibility is that the regulation of *PLAB* gene expression is disordered as part of the malignant phenotype. This possibility will be examined in a later section.

Because *GDF-1* expression was widely found, we determined whether its expression could be modulated in response to inducers (Fig. 4). Treatment of Reh cells with PMA results in a 6-fold ($p < 0.01$) decrease in the amount of *GDF-1* message detected. By contrast, no significant difference in *GDF-1* message was detected on the induction of erythroid differentiation of TF1 cells with ALA.

Since we could detect the expression of *BMP-2* in normal marrow, the effect of TPA treatment on the expression of *BMP-2* expression in Reh cells was measured (Fig. 5). A 2-fold ($p < 0.01$) increase in *BMP-2* message was seen.

As it has recently been reported that *BMP-4* message can be detected in normal monocytes by reverse transcription and PCR (34), we treated the following cell lines with inducers of monocyte/macrophage differentiation and assayed for the presence of *BMP-4* message by the RNase protection assay: TF-1 treated with TPA, U937 treated with TPA or retinoic acid and TPA, and Reh treated with TPA. No *BMP-4* message was found (data not shown).

Table 1 summarizes the result of the screen for expression genes of the transforming growth factor-beta superfamily in hematopoietic cell lines, normal bone marrow, and circulating monocytes, B cells and T cells. The results of the differentiation experiments are also included. Of the nine genes studied, four exhibit a lineage-limited pattern of expression. *BMP-4* and *BMP-7* expression is predominantly lymphoid, while the expression of *PLAB* and *GDF-1* is predominantly myeloid. The presence of *GDF-1* message in Reh cells is as consistent with a myeloid function as a lymphoid one, given the biphenotypic potential of the Reh cells. However, the detection of *GDF-1* expression in Jurkat, a mature T-lymphoid cell line, and the detection of *BMP-4* expression in normal bone marrow argue that these genes will prove to have multiple roles in hematopoietic development.

Levels of BMP Message

A marked variation was found in the amount of *PLAB*, *BMP-7*, and *BMP-4* message in the cell lines, with some cell lines expressing high levels of message (>20 times) the level of message found in the corresponding normal hematopoietic tissue. In normal hematopoietic tissue, message was either undetectable or at the lower limit of detectability by the RNase protection assay.

Treatment with TPA activated *PLAB* expression in Reh and U937 cells. The amount of message produced was about 3-fold greater in U937 than in Reh cells (Table 2). These results are consistent with a recently proposed model (38) in which *PLAB* is secreted by activated

Table 2. Relative Levels of *PLAB* Message in Hematopoietic Cells

TF-1	K562	K562 + 24 hr TPA	Reh + TPA	U937 + TPA
(1)	63 +/- 16	168 +/- 33	4.6 +/- 1.1	14.7 +/- 2.5

Note. Total RNA (50 µg/sample) was analyzed by the RNase protection assay. Data were collected by multiple autoradiographic exposures and analyzed by the NIH Image program or were collected with a Phosphorimager and analyzed by the accompanying software. Variations in loading were controlled for by comparison with the signal for 18S RNA. The amount of TF-1 message was defined as 1. Results represent the mean +/- the standard deviation of two to four separate experiments.

macrophages as part of a feedback mechanism to dampen macrophage activation. Part of the evidence cited in support of this model is that *PLAB* message is found more abundantly in activated cell lines representative of later stages along the monocyte/macrophage developmental pathway than cell lines representative of earlier stages. As Reh cells exhibit lymphoid features and retain the capacity to differentiate along the macrophage pathway, Reh cells thus display some of the features of the hypothetical lymphoid/myeloid stem cell. U937 cells, being committed to monocyte differentiation are further down the monocytic developmental pathway than Reh cells, and activated U937 cells produce more *PLAB* message than do activated Reh cells. The amount of *PLAB* message produced by activated TF-1 cells is roughly comparable to that produced by activated Reh cells, which is consistent with this model. TF-1 cells may be considered similar to the myeloid stem cell, and therefore would be expected to produce less *PLAB* message when activated than U937. The level of *PLAB* message produced by K562 cells is inconsistent with this model. TF-1 cells and K562 cells are similar in their degree of differentiation; presumably, they would produce comparable amounts of *PLAB* message.

However, K562 cells produce 60-fold greater *PLAB* message than TF-1 cells. TPA-induced K562 cells produce 11 times more *PLAB* message than activated U937 cells. This result suggests that the regulation of the production of *PLAB* message in K562 cells is aberrant.

BMP-7 expression may be deregulated in the B-lymphocytic cell line Ramos. Compared with normal circulating B cells, Ramos produces 45-fold more *BMP-7* RNA (Table 3). As Ramos is a mature B-cell line, it seems unlikely that the high level of *BMP-7* expression represents normal *BMP-7* expression at that point in the developmental pathway.

BMP-4 expression may be deregulated in T-cell lines. Three out of three T-cell lines tested expressed the *BMP-4* gene, albeit at widely varying levels (Table 4). We were unable to detect *BMP-4* expression in circulating T cells by the RNase protection assay, suggesting that the normal amount of *BMP-4* is quite small.

DISCUSSION

Our results suggest that individual bone morphogenetic proteins may be involved in the development and function of specific hematopoietic lineages. The elevated expression of BMP genes found in some leukemia cell lines suggests that aberrant BMP signaling is a feature of some hematopoietic malignancy.

BMP signaling is transduced through transmembrane serine-threonine kinase receptors, classified as Type I and Type II (40). A complex is formed consisting of the BMP, Type I receptor, and Type II receptor, followed by phosphorylation of the Type I receptor by the Type II receptor kinase domain. The Type I receptor then phosphorylates a member of the

Table 3. Relative Levels of *BMP-7* Message in Hematopoietic Cells

B cells	T cells	Reh	Ramos	Molt4	Sup-T1	Jurkat
(1)	0.75	Not seen	45	Not seen	Not seen	Not seen

Note. Total RNA (50 µg/sample) was analyzed by the RNase protection assay. Data were collected by multiple autoradiographic exposures and analyzed by the NIH Image program. Variations in loading were controlled for by comparison with the signal for 18S RNA. The intensity of the B-cell signal was defined as 1. Results represent 2 separate experiments.

Table 4. Relative Levels of *BMP-4* Message in Hematopoietic Cells

Bone marrow	T cells	Molt-4	Sup-T1	Jurkat
(1)	Not seen	8.0 +/- 3.9	187 +/- 67	23 +/- 14

Note. Total RNA (50 µg/sample) was analyzed by the RNase protection assay. Data were collected with a Phosphorimager and analyzed by the accompanying software. Variations in loading were controlled for by comparison with the signal for 18S RNA. The intensity of the bone marrow signal was defined as 1. Results represent the mean +/- the standard deviation of three to four separate experiments.

SMAD protein family. The activated SMAD associates with a non-phosphorylated co-SMAD, and the complex translocates to the nucleus where it interacts with transcription factors to regulate gene expression. The BMPs, the receptors, and the SMADs are all gene families with multiple members, which ensures many possible combinations of regulatory interactions.

Members of the BMP family have been shown to have antiproliferative effects on their target cells (41, 42). If the same is true of the hematopoietic system, then disruption of the BMP signaling pathways may play a role in the development of leukemia and lymphoma. Disruption of BMP signaling pathways has been found in malignancies of non-hematopoietic tissues. *SMAD4/DPC4* frequently is deleted or mutated in pancreatic carcinomas (43) and colorectal carcinomas (44). Although an extensive survey of tumors (45) has found that, aside from pancreatic and colorectal cancer, *SMAD4* mutations are rare, it should be noted that no hematopoietic malignancies were included in the study. Thus, mutations in *SMAD4* have not been eliminated as contributing factors in the development of leukemia and lymphoma. Another member of the BMP signaling cascade, *SMAD5*, is found at the proposed site of a tumor suppressor gene in myeloid leukemia (46). Incubation of hematopoietic progenitor cells with antisense oligonucleotides to *SMAD5* reverses the inhibitory effect of TGF-β on colony formation (47).

Mutations in the BMP receptors are contributory agents to the development of cancer. Mutations in the TGF-β Type II receptor are

common in colon cancer (48) and in squamous head and neck carcinomas (49); mutations in the TGF-β Type I receptor have been found in prostate, colon, and gastric cancer (50). TGF-β receptor defects have been found in B- and T-lymphoid malignancies (51, 52) and loss of sensitivity to TGF-β has been correlated with disease progression.

As a result of the block in TGF-β signal transduction, levels of TGF-β mRNA might be increased in a fruitless effort to halt uncontrolled proliferation. Such increases in TGF-β message have been detected in breast, prostate, and bladder cancer (53-55). The marked elevation of *BMP-7* message in the Ramos cell line, of *PLAB* message in the K562 cell line, and of *BMP-4* message in the SupT1 cell line are consistent with such a mechanism. If so, then analysis of the proteins involved in the individual bone morphogenetic signaling pathways might lead to the identification of new molecular lesions important in the development of hematopoietic malignancy.

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