A signaling mechanism for growth-related expression of fetal hemoglobin

Natarajan V. Bhanu, Tiffany A. Trice, Y. Terry Lee, and Jeffery L. Miller

Increases in fetal hemoglobin have been identified after birth in several clinical settings associated with stressed or malignant erythropoiesis. To better understand the relationship between the expression of this fetal protein and growth, donated human erythroid progenitor cells were cultured in the presence of erythropoietin (EPO) plus the growth-modifying cytokine stem cell factor (SCF), and several growth-related signaling pathways were interrogated. Only the MEK1/2 inhibitor (PD98059) demonstrated significant effects on fetal hemoglobin. In the absence of PD98059, levels of fetal hemoglobin averaged 27.4% ± 7.9% in EPO+SCF compared with 1.26% ± 1.7% in EPO alone ($P = .02$). A linear dose response in levels of fetal hemoglobin to PD98059 was detected (0.16 μM = 27.13%, 0.8 μM = 19.6%, 4 μM = 12.2%, 20 μM = 1.54%). Western blot analyses revealed that SCF was required for phosphorylation of MEK and p44MAPK in this setting, and quantitative polymerase chain reaction demonstrated a significant increase in γ-globin mRNA. Particular perturbations of growth-related signaling may also function to activate tissue-specific genes normally expressed during fetal development. This concept may be relevant for the development of new treatment rationales for beta hemoglobinopathies. (Blood. 2004;103:1929-1933)

Introduction

The persistence of unusually high levels of fetal hemoglobin beyond birth is known to alleviate the clinical severity in beta hemoglobinopathies.[1] For this reason, a decades-long search for therapeutic modulators of fetal hemoglobin (HbF) has continued to motivate basic and clinical investigators alike.[2] As a result, several pharmacologic agents have been shown to augment HbF production. Included are cytotoxic drugs thought to have direct effects on γ-globin gene transcription as well as indirect effects mediated through increased erythroid progenitor cell proliferation as part of a rebound or regeneration effect.[3,4] Increases in HbF are also associated with recovery from marrow ablation in the setting of bone marrow transplantation.[5] HbF is also considered to be an oncofetal protein due to higher levels associated with erythroleukemias, myelodysplastic syndromes, and juvenile myelomonocytic leukemia[6] (JMML). In each of these cases, increases in HbF correlate with perturbations of progenitor cell growth.

Correlations between HbF and growth have also been reported in studies involving erythroid-directed cytokines.[7] Erythropoietin (EPO) and other signaling factors have demonstrable effects on the survival, proliferation, and differentiation of erythroblasts. These effects may result due to interplay between intracellular signals[8,9] and specific transcriptional regulators.[10] EPO augments HbF after acute intravenous administration, but that effect is lost after chronic use.[4] Stem cell factor (SCF) has also been shown to regulate erythroid growth and HbF production in vivo and in primary cells.[11-14] Among cultured human cells from cord blood and adults, SCF has considerable HbF-inducing ability at the proerythroblast stage of differentiation that correlates well with the expression of CD117.[15] SCF binding results in the autophosphorylation of its receptor (CD117) and creates binding sites for SH2 domain–containing proteins, including those of the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway. SCF- and EPO-mediated signals interact at various points along the Ras/Raf1/MAPK and PI3K pathways to affect specific biologic responses.[8,9] Here we explore the hypothesis that growth-related signaling pathways may coincidentally regulate HbF synthesis during adult human erythropoiesis.

Materials and methods

Primary erythroblast cultures

After obtaining informed consent, human $CD34^+$ cells were isolated in high purity from the peripheral blood of healthy human volunteers. The cells were cultured at a concentration of 10^4 cells/mL to 10^5 cells/mL in medium supplemented with 4 U/mL EPO (Amgen, Thousand Oaks, CA) and 50 ng/mL SCF (R&D Systems, Minneapolis, MN) as described previously.[13] Matched cultures were incubated for 14 days in increasing concentrations of inhibitors for different signaling pathways: AG490 for the JAK2 pathway,[16] GF109203X for the PKC pathway,[17] SU6656 for Src protein kinase,[18] wortmannin for PI3K,[9] PD98059 for the MEK and p44/p42MAPK pathways,[9] SB203580 for the p38 MAPK pathway,[19] as well as ODQ, LY83583, and NS2028 for the guanylate cyclase pathway.[20] All inhibitors were purchased from Calbiochem (La Jolla, CA), except for PD98059 and SB203580 (LC Laboratories, Woburn, MA). Cells were enumerated using an electronic cell counter (Coulter, Hialeah, FL).

HPLC for adult and fetal hemoglobin

Aliquots of one million cells were washed in Dulbecco phosphate-buffered saline (PBS; CellGro, MediaTech, Herndon, VA). The pellets were lysed by repeated freeze-thaw and analyzed for HbF and adult hemoglobin (HbA) content, using a 20-mm × 4-mm POLYCATA column (Poly LC, Columbia, MD) fitted to a Gilson high-performance liquid chromatography (HPLC) system (Gilson, Middleton, WI) as previously described.[13] The HbF and HbA retention times were validated by electrospray mass spectroscopy.[13]

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Western blotting

For analyzing the phosphorylation pattern of the protein kinases MAPK and MEK, cells were grown in EPO-containing Dulbecco modified Eagle medium (DMEM) for 6 days. The cells were then harvested at 0, 10, and 30 minutes after the addition of 50 ng/mL SCF. Extracted protein was prepared using Protein Extraction Reagent (M-PER) (Pierce Biotechnology, Rockford, IL) as recommended by the manufacturer, electrophoresed (25 μg/lane) through sodium dodecyl sulfate (SDS)–polyacrylamide gel, transferred to nitrocellulose, and probed for total and phosphorylated forms of MEK1/2 and p44/p42MAPK. The primary antibodies were obtained from Calbiochem (San Diego, CA) and Cell Signaling Technology (Beverly, MA), respectively. Secondary horseradish peroxidase (HRP)–conjugated antibodies were purchased from Amersham Pharmacia (Piscataway, NJ).

Quantitative PCR for γ- and β-globin expression

SCF was added to $CD34^+$ hematopoietic progenitor cells grown in EPO for 6 days. RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) on days 0, 2, 4, 6, and 8, after SCF stimulation and quantitative PCR performed as described earlier.[13] All statistical determinations of significance were determined by Student paired *t* test analysis.

Results

The MEK inhibitor, PD98059, has a dose effect on SCF-induced HbF expression in cultured primary human erythroblasts

In the presence of EPO or EPO+SCF, human progenitor cells underwent a gradual and homogeneous wave of erythroid differentiation and maturation, which at day 14 was composed of a major population of predominantly GPA^+ nucleated erythroblasts. We have previously shown that supplementing SCF to the growth medium resulted in proliferation and increased HbF to levels greater than those achieved in EPO alone.[15] In the present study, a 10-fold increase in the number of erythroblasts was detected in the presence of SCF. The HbF/HbA+HbF ratio averaged 27.4% ± 7.9% in EPO+SCF compared with 1.26% ± 1.7% in EPO alone ($P = .02$).

Using this culture system, we used signaling pathway inhibitors to further explore possible relationship(s) between erythroid growth and HbF in response to SCF. We considered several pathways, including those associated with binding of EPO, SCF, or both ligands to receptors on the erythroblast surface. Interrogated pathways included JAK2 (inhibitor: AG490),[16] PKC (inhibitor: GF109203X),[17] phospho-inositol 3 kinase (PI3K; inhibitor: wortmannin),[9] Src (inhibitor: SU6656),[18] MEK (inhibitor: PD98059),[9] and p38 MAPK (inhibitor: SB203580).[19] Recently, the guanylate cyclase pathway has been shown to be important in induction of γ-globin in erythroleukemic cells and primary erythroblasts by butyrate and hemin[20] as well as by hydroxyurea and CysNO.[21] Therefore, we additionally evaluated the effect of inhibitors, ODQ, LY83583, and NS2028 to explore the possible involvement of soluble guanylate cyclase in SCF-mediated increase in HbF. For each inhibitor, matched cultures containing EPO versus EPO+SCF were performed with increasing levels of inhibitors added to the culture medium. Serial titrations (1:5) above and below the inhibitory concentrations (IC_{50}) provided by the manufacturers were performed for each inhibitor over a 2- to 3-log range. For all inhibitors, the concentrations were titrated above the IC_{50} to levels consistent with those reported elsewhere.[9,16-20] The concentration ranges were as follows: AG490 (0.032-4 μM), GF109203X (0.002-1 μM), SU6656 (0.16-500 nM), wortmannin (0.064-40 nM), PD98059 (0.032-20 μM), SB203580 (0.01-2 μM), ODQ (0.08-250 μM), LY83583 (0.04-125 μM), and NS2028 (0.02-62.5 μM). Of note, titration of GF109203X, PD98059, AG490, and wortmannin to even higher levels completely inhibited the production of hemoglobin-producing cells over the 14-day culture period.

Inhibitors for JAK, Src, MEK, p38 MAPK, PI3K, PKC, and cGMP pathways produced no significant change in HbF/HbA+HbF in cells grown in EPO and EPO+SCF after 14 days (Figure 1A). Similarly, dimethyl sulfoxide (DMSO; vehicle control; 0.06%-0.2%) had no significant effects on HbF. In distinct contrast to the other inhibitors, the MEK inhibitor, PD98059, had a significant effect on the HbF content in primary human progenitor cells grown in EPO+SCF for 14 days. Little effect was detected at PD98059 concentrations of 0.16 μM and lower (HbF/HbA+HbF was 27.4% ± 8.0% in EPO+SCF compared with 1.26% ± 1.7% in EPO alone). However, increasing the concentration of PD98059 to levels above 0.16 μM resulted in a linear, dose-dependent lowering of HbF/HbA+HbF. This effect became statistically significant both at 4 μM (12.2% ± 6.2%) and 20 μM (1.54% ± 1.42%), and the HbF/HbA+HbF receded to a percentage equivalent to that in cells grown in EPO alone (Figure 1B,D). Interestingly, the significant HbF dose effects of PD98059 at concentrations of 4 μM to 20 μM were not accompanied by equivalent reductions in cell numbers ($1.4 \times 10^6 \pm 1.2 \times 10^6$ cells in Epo+SCF plus 20 μM PD98059 compared with $1.7 \times 10^6 \pm 5.7 \times 10^5$ cells in Epo+SCF; $P = .52$; Figure 1C). At even higher concentrations (100 μM PD98059), proliferation was completely inhibited, and hemoglobin-producing cells were not detected.

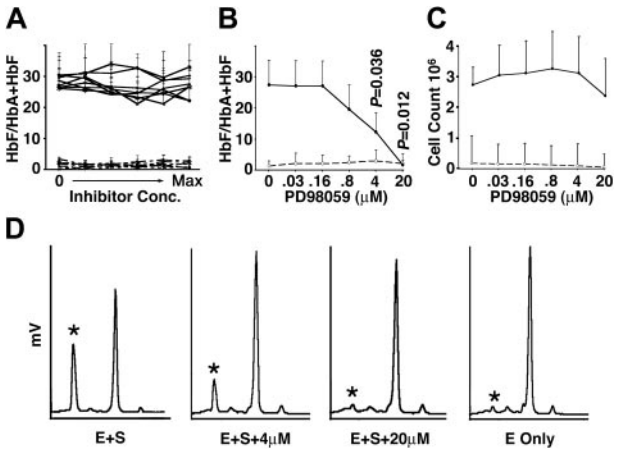

Figure 1. MEK inhibitor PD98059 blocks SCF-induced HbF production. (A) HPLC was performed on 1.5 million cells grown for 14 days in EPO-containing medium (4 U/mL) or EPO+SCF–containing medium (50 ng/mL), treated with increasing concentrations of various inhibitors. The HbF/HbA+HbF ratios are expressed as a percentage for EPO+SCF (upper solid lines) and EPO (lower dashed lines) for each inhibitor and shown with standard deviation bars (experiments performed in triplicate with cells from 3 separate donors). Points on the x-axis represent final concentrations for each inhibitor defined by (1:5) serial dilutions from a maximum concentration (Max) compared with cultures containing no inhibitors (0). Symbols and maximum concentration: AG490 (asterisk), max = 4 μM; LY83583 (plus sign), max = 125 μM; SU6656 (bar), max = 500 nM; NS2028 (square), max = 62.5 μM; GF109203X (cross), max = 1 μM; ODQ (diamond), max = 250 μM; SB203580 (circle), max = 2 μM; and wortmannin (triangle), max = 40 nM. (B) PD98059 effects on HbF/HbA+HbF. The format is the same as for panel A, except that the exact concentrations of PD98059 are shown for each serial dilution on the x-axis. *P* values demonstrating a significant reduction ($P < .05$) at 4 μM and 20 μM are shown and represent a comparison with cells cultured in the absence of PD98059. (C) PD98059 effects on cell count shown for comparison. (D) HPLC patterns showing hemoglobin peaks (mV) (HbF peaks marked by asterisk) of a representative experiment. E+S indicates culture containing EPO+SCF without PD98059; E+S+4 μM indicates culture containing EPO+SCF plus 4 μM PD98059; and E only, culture containing EPO alone (ie, without PD98059).

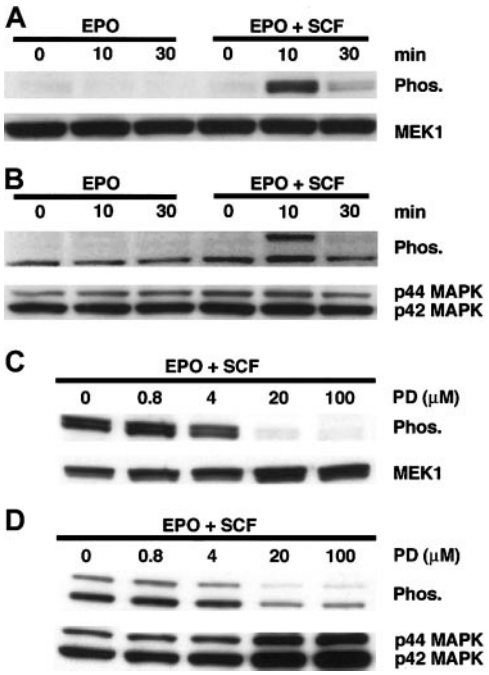

Figure 2. SCF specifically activates MEK and p44/p42MAPK in a time-dependent manner. Hematopoietic progenitor cells cultured in EPO-containing medium for 6 days prior to the addition of SCF. Cellular protein was analyzed by Western blotting at 0, 10, and 30 minutes after the addition of SCF (EPO+SCF) and compared with protein extracts from matched controls (EPO). (A) Phosphorylated (Phos) and total MEK1 (lower bands) as well as (B) phosphorylated and total p44/p42MAPK were studied. (C) Hematopoietic progenitor cells cultured in medium containing EPO and varying concentrations of PD98059 (PD; 0.8-100 μM). Cell protein extracts were probed 10 minutes after the addition of SCF. Phosphorylated and total MEK as well as (D) phosphorylated and total p44/p42MAPK bands are shown for comparison.

Activation of MEK and p44MAPK by SCF

Our inhibitor studies suggested that PD98059, a specific inhibitor of the kinase activity of MEK on p44/p42MAPK, blocked the SCF effect on HbF/HbA+HbF (Figure 1B,D). To further investigate SCF-mediated activation of these signal proteins, we performed time-course studies of phosphorylation. We examined cells in this context after 6 days in culture, based on the high levels of CD117 and related SCF effects identified at the proerythroblast stage of erythroid development.[15] Phosphorylation analyses were performed on cell extracts obtained 0, 10, and 30 minutes after addition of SCF. Western blot analyses were performed using antibodies for phosphorylated and total proteins. Cells cultured in the absence of SCF showed no MEK activation (Figure 2A). At 10 minutes after SCF addition, phosphorylated MEK was clearly demonstrated, followed by dephosphorylation after 30 minutes. Western analyses of p44MAPK and p42MAPK, the main downstream targets of MEK, were also performed. Phosphorylation revealed that p42MAPK was constitutively activated, possibly by EPO or serum factors in the culture medium (Figure 2B). The phosphorylation of p44MAPK (Figure 2B) was in concert with MEK activation, detected at 10 minutes after SCF addition. Hence, SCF addition resulted in activation of MEK and related activation of p44MAPK additive to that of p42MAPK detected in EPO alone.

Western analyses were also performed to determine whether PD98059 inhibited MEK and MAPK under the conditions described. Cells were cultured in EPO medium containing increasing concentrations of PD98059 (0.8-100 μM). On culture day 6, 10 minutes after the addition of SCF, protein extracts were obtained for MEK and p44/p42MAPK activation assays. The MEK and MAPK phosphorylation patterns in cells grown in the presence of PD98059 are shown in Figure 2C-D. MEK and p44/p42MAPK phosphorylation were clearly detected at the lower concentrations of PD98059. When cultured in 4 μM PD98059, the cells' ability to phosphorylate MEK in response to SCF was maintained at a slightly lower level according to this assay. However, at the higher concentrations of PD98059 (20 μM and 100 μM), inhibition of MEK was complete with the phosphorylated protein detectable only at background levels. The pattern for p44/p42MAPK was consistent with that of MEK, but low levels of phosphorylated p42MAPK were present even at the higher concentrations of PD98059 (Figure 2D). The DMSO vehicle (maximum concentration 0.2%) for PD98059 had no effect on SCF-mediated MEK/MAPK activation (data not shown).

Analyses of β- and γ-globin transcripts

p44MAPK and p42MAPK activate several transcription factors, such as CREB, ATF-2, and Elk-1, to stimulate expression of a number of immediate early genes.[22] These kinases also regulate the expression of some proteins at the posttranslational level.[23,24] Therefore, in order to elucidate the mechanism whereby MEK/p44MAPK leads to increased HbF, we quantified the β- and γ-globin transcripts using real-time PCR (Figure 3A-C). On day 6, SCF was added to the culture medium and mRNA was collected on subsequent days and compared with controls grown in the absence of SCF. Our results indicated that there was a significant change in the pattern of γ-globin gene transcription in the presence of SCF (Figure 3A). In EPO alone, the number of γ-globin transcripts per cell peaked at around 1000 molecules/cell on day 8. When SCF was added on day 6, γ-globin transcripts continued to rise to a peak of more than 5000 transcripts/cell on days 10 and 12. This rise was statistically significant by day 12 ($P = .02$). In contrast, the average

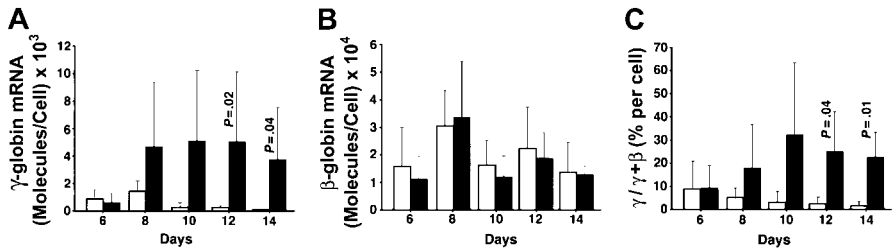

Figure 3. Quantitation of β- and γ-globin by amplification of mRNA from human hematopoietic progenitor cells. CD34+ cells grown in EPO for 6 days were stimulated by SCF and RNA extracted from cells harvested on days 6, 8, 10, 12, and 14 were amplified by quantitative PCR. (A) Average number of γ-globin molecules per cell. (B) Average number of β-globin molecules per cell. (C) Average γ/γ+β percentages in SCF-stimulated cells (solid bars) were compared with matched controls cultured in EPO alone (open bars). The panels show values averaged from 3 donors performed in separate experiments. Standard deviation bars are shown by vertical lines. The significant *P* values are shown and represent a comparison between EPO+SCF versus EPO.

β-globin mRNA/cell in EPO+SCF demonstrated a pattern similar to that found in the EPO controls (Figure 3B). While the β-globin mRNA/cell was slightly lower in EPO+SCF on days 10 to 14 compared with EPO, those differences did not achieve statistical significance. As a result of the increased γ-globin and decreased β-globin levels, the shift in γ/β+γ-globin mRNA by day 14 is similar to that of the protein profile represented as HbF/HbA+HbF% (compare Figures 1 and 3).

Discussion

In this study, we systematically explored donated peripheral blood–derived progenitor cells cultured in single phase to better understand the relationships between erythropoiesis and globin gene regulation. SCF supplementation in cultures resulted in both increased cell proliferation as well as HbF production.[12,15] Herein, we determined that inhibition of MEK resulted in a dose-related reversal of SCF-mediated increases in HbF. Notably, the dose of PD98059 required for a significant reversal of the HbF effect was lower than that associated with significant growth inhibition. Further analyses revealed that activation of MEK resulted in p44MAPK phosphorylation that was not detected in cultures supplemented with EPO alone, and that the increases in HbF were achieved primarily through increases in γ-globin gene transcription. These results suggest that SCF binding to the cell surface results in MEK phosphorylation which leads to increases in γ-globin gene transcription and HbF. Whether this signaling pathway mediates direct epigenetic modifications within the globin gene locus or acts through indirect cellular and biochemical events is currently unknown. The increases in γ-globin transcription are unlikely to be limited to rare subpopulations of cells since the SCF-induced increases in HbF were detectable in a majority of cells.[15] Of note, our screening assay provided no evidence that other signaling pathways were active in SCF-mediated HbF modulation, including the soluble guanylate cyclase pathway previously reported to increase γ-globin production using other HbF inducers.[20,21] While MEK activation appears to be required for SCF-mediated HbF modulation, cross talk or signaling redundancy may have masked a possible role for other pathways in the modulation of HbF. Interestingly, sodium butyrate,[25] TGF-beta,[26] nitric oxide,[27] soluble guanylate cyclase,[27] and cAMP[28] can inhibit growth or have differential (activation or inhibition) effects on MEK or MAPK. These stimuli may have activities in erythroblast signaling distinct from SCF.

MEK is a signaling node for growth and γ-globin transcription in adult erythroblasts. The Ras/Raf/MEK/p44/p42MAPK cascade constitutes a central, functional signaling module that links surface receptor–mediated signals to nuclear events in erythroblasts. After serum starvation, addition of EPO and SCF activates MEK and p44/p42MAPK in human erythroblasts in order to increase proliferation.[8,9] Our kinase phosphorylation experiments included SCF addition as the single experimental variable to avoid confounding issues associated with serum starvation. Under these conditions, we determined that MEK and possibly p44MAPK are the primary mediators for SCF-induced increases in HbF. At higher concentrations of PD98059, this pathway was also shown to be necessary for erythroid growth and associated hemoglobinization. PD98059 is highly specific for insulating the signal transduction from Raf1 to MEK1.[29] While p42MAPK phosphorylation was detected in the absence of SCF, the phosphorylation of p44MAPK was SCF dependent. Although p44MAPK and p42MAPK are usually coactivated, they are coded by discrete, evolutionarily conserved genes that may be functionally distinct.[30] Upon activation, p44MAPK and p42MAPK molecules homodimerize, translocate to the nucleus, and regulate the activity of many transcription factors. Recently, a number of transcription factors, including TAL1 and FKLF, were shown to be expressed in SCF-mediated up-regulation of HbF.[31] Further investigation will be required to determine whether MEK increases γ-globin transcription through these factors, epigenetic modifications, or other mechanism(s).

Based on the data described here and elsewhere, we propose a molecular mechanism that links signaled modifications of erythroid growth with the modulation of fetal hemoglobin (Figure 4). At steady state, EPO signaling results primarily in cell survival and associated β-globin gene transcription in adult erythroblasts. EPO activates JAK2 in erythroid cells to increase cell survival through a mechanism involving STAT5 or other molecules.[32,33] We propose that some conditions like erythroid stress, regeneration, or neoplasia result in activation of signaling that serves a role in modifying the growth of the erythroid progenitor cells as well as increasing γ-globin transcription in those cells. In the case of SCF, these effects may be achieved through MEK activation and resulting p44MAPK phosphorylation and other downstream events. As evidenced by the nearly complete reversal of HbF production and MEK phosphorylation in 20 μM to 100 μM PD98059, activation of this pathway appears to be required for the HbF-modulating effects of SCF. However, PD98059 at a lower concentration (4 μM) significantly reduced the SCF effect on HbF, but this concentration had little effect on growth. In addition, the ability of the cells cultured in 4 μM PD98059 to phosphorylate MEK or MAPK in response to SCF suggests that complete inhibition of the pathway is not required for a reduction in HbF to be manifest. Alternatively, PD98059 may have novel effects on γ-globin gene expression that are not exclusively mediated by MEK. These results suggest that SCF modulation of HbF may involve signaling kinetics or downstream events that are not identical to those that modify growth. While not studied here, it should not be overlooked that growth-related signaling cascades may be involved in the regulation of ontogeny-defined events such as hemoglobin switching.

Our findings may be more generally relevant toward understanding the nature of fetal antigen expression in some leukemias. Decades ago, an intriguing association between JMML (previously called juvenile chronic myeloid leukemia, JCML) and elevated levels of HbF was recognized.[34] Later, it was discovered that several patients with JMML harbor mutations in neurofibromin (NF1), a negative regulator of Ras[35] (Figure 4). In hematopoietic progenitor cells, constitutive activation of Ras/MAPK signaling

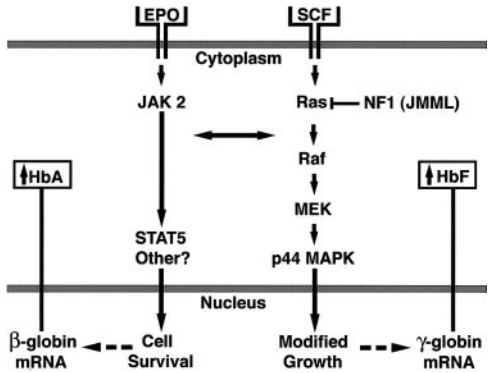

Figure 4. Signaling network of SCF-mediated HbF augmentation.

secondary to the absence of NF1 results in MEK and p42/p44MAPK activation.[36] Based on our results, we propose that constitutive activation of MEK in the cells of patients with JMML with NF1 mutations may have a central role in re-emergence of HbF. A variety of embryonic or fetal restricted genes have been identified in tumors.[37] These oncofetal antigens all share the biologic features of cancer-associated expression of tissue-restricted genes that are normally detected in the early stages of human life. At least some of these antigens may arise through cancer-related perturbations of signaling cascades. If growth-related signaling activates the expression of fetal genes, then manipulation of growth-related signaling may have clinical utility. Inhibitors of the relevant kinases or other signaling molecules might be considered in patients with leukemias or other cancers marked by the expression of embryonic or fetal genes. In contrast, molecules designed for controlled activation of kinases such as MEK might be envisioned to increase expression of HbF in patients with sickle cell disease or β-thalassemia.

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