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Oncostatin M-Mediated Regulation of KIT-Ligand-Induced Extracellular Signal-Regulated Kinase Signaling Maintains Hematopoietic Repopulating Activity of Lin$^-$CD34$^+$CD133$^+$ Cord Blood Cells

ROBERT A.J. OOSTENDORP,[a] SIV GILFILLAN,[a] AMANDA PARMAR,[a] MATTHIAS SCHIEMANN,[b,f] STEFANIE MARZ,[a]
MARKUS NIEMEYER,[c] SABINE SCHILL,[d] EDELBURGA HAMMERSCHMID,[e] VOLKER R. JACOBS,[c]
CHRISTIAN PESCHEL,[a] KATHARINA S. GÖTZE[a]

[a]3rd Department of Internal Medicine, [b]Department of Microbiology and Immunology, [c]Department of Obstetrics and Gynaecology, [d]Department of Radiation Oncology, Klinikum rechts der Isar, Technische Universität München, Munich, Germany; [e]Institute for Experimental Oncology and [f]Clinical Cooperation Group "Antigen-specific Immunotherapy," Helmholtz Zentrum München, Munich, Germany

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ABSTRACT

We investigated whether KIT signaling was sufficient to maintain human hematopoietic stem cells in culture or whether, as with murine stem cells, signaling through glycoprotein 130 (gp130) is additionally required. Sorted CD34$^+$ CD133$^+$(CD33/CD38/CD71)$^-$ cells from human umbilical cord blood (UCB) were cultured in the presence of combinations of KIT-ligand (KL) and the gp130 stimulating molecule oncostatin M (OSM). We found that OSM increased KL-induced proliferation, which was accompanied by an expansion in numbers of mature progenitors colony-forming cells (CFC, CAFCw2). More primitive progenitors, CAFCw6 and long-term culture-CFC, were not maintained by KL as a single factor. Although addition of OSM did not improve survival, the KL/OSM combination showed improved maintenance of immature progenitors as well as higher CD34 expression. Similarly, both KL and OSM were required to maintain NOD/SCID-repopulating activity. In experiments to investigate the underlying mechanism, we found that extracellular signal-regulated kinase (ERK) and its downstream target p90 ribosomal S6 kinase were activated by KL and downregulated by the inclusion of OSM during stimulation. The p38 mitogen-activated protein kinase (p38 MAPK) was not modulated by either KL or OSM. Indeed, many of the effects of OSM (increased cell division, maintenance of CFC, and maintenance of high CD34 expression) could be mimicked by using the mitogen-activated protein kinase kinase inhibitor U0126. More importantly, NOD/SCID-repopulating activity was preserved in the KL/U0126-stimulated cells, but not in cells stimulated with a combination of KL and the p38 MAPK inhibitor SB203580. Our results show that the loss of repopulating activity during KL stimulation is counteracted by OSM through the downregulation of ERK pathway signaling. STEM CELLS *2008;26:2164–2172*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Hematopoietic stem cells (HSC) maintain the blood system throughout life. To achieve this, HSC remain quiescent and are recruited to undergo self-renewal and differentiating divisions under appropriate stimuli. In the last couple of years, much has become clear about the mechanisms of quiescence. In particular, the hematopoietic stem cell niche plays a central role in dampening cell cycle progression of hematopoietic and other stem cells [1]. However, much less is known about which stimuli are required to recruit HSC into cell cycle or which stimuli are involved in maintaining HSC identity through self-renewal divisions. The observations that HSC localize to vascular and endosteal regions of the adult bone marrow suggest that other cells in these niches are involved in HSC regulation. Although it is suspected that the most important role for the niche is to keep the HSC quiescent, hematopoietic niches also produce many factors capable of recruiting HSC into cell cycle [2].

One of the earliest identified factors is stem cell factor, which is present in both membrane-bound and soluble variants [3]. Stem cell factor was found to be the ligand for the KIT tyrosine kinase (KL, [4, 5]), which is expressed by hematopoietic stem cells [6]. For this reason, many investigators have included KL in protocols

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Correspondence: Robert A.J. Oostendorp, Ph.D., Laboratory of Stem Cell Physiology, III. Medizinische Klinik und Poliklinik, Klinikum rechts der Isar, Ismaningerstrasse 22, 81675 Munchen, Germany. Telephone: +49 89 4140 6056; Fax: +49 89 4140 6057; e-mail: oostendorp@lrz.tum.de Received December 11, 2007; accepted for publication May 14, 2008; first published online in STEM CELLS *EXPRESS* May 22, 2008. ©AlphaMed Press 1066-5099/2008/$30.00/0 doi: 10.1634/stemcells.2007-1049

aimed at expanding hematopoietic progenitors as well as HSC. KL by itself will induce proliferation of late progenitors, but to promote stem cell expansion, KL requires additional growth factors [7]. KL has been shown to synergize with many different growth factors, including thrombopoietin (TPO), Flt3-ligand (FL), granulocyte colony-stimulating factor (G-CSF), and cytokines, which signal through the gp130 coreceptor such as interleukin (IL) six (IL6), IL11, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and leukemia inhibitory factor (LIF) [7–10].

In mice, the gp130 signal-transducing unit of the IL6 family of cytokines was shown to be necessary for proper development of definitive hematopoiesis [11]. Furthermore, it was demonstrated that signaling through the gp130 receptor chain of the IL6 family of cytokines was important for HSC self-renewal [12]. Which IL6 family of cytokines delivers the gp130 signal in vivo is unclear. It seems unlikely that IL6 itself is this mediator, as the IL6R$^-$CD34$^+$ cells are the most clonogenic cord blood cells [13]. A combination of IL6 and its soluble receptor [14] or a designer molecule fusing IL6 and its soluble receptor (H-IL6, [15]) stimulates gp130 independently of the cytokine-specific receptors, and thus can bypass the necessity of the presence of a specific receptor. Indeed, both the IL6/sIL6 receptor(R) complex and H-IL6 promote self-renewal of HSC in combination with other cytokines [16–19].

Specificity of the gp130 cytokine family depends on a cytokine-specific receptor. It has been described that stimulation with oncostatin M (OSM), the IL6/IL6R complex, or LIF does not necessarily have the same signaling outcome [20]. Indeed, in the defective myeloid compartment in Stat4−/− mice could be restored by treating the mice with OSM, but not IL6 [21]. In the present manuscript, we investigate OSM-mediated effects on human hematopoietic cells. OSM is a 36-kDa member of the IL6 family of cytokines. OSM interacts with at least three types of receptors: the OSMR/gp130, the LIFR/gp130, and the recently identified OSMR/IL31RA heterodimeric receptors. Mice deficient in expression of OSMR mainly show defects in megakaryo- and erythropoiesis, which is due to both loss of microenvironmental support and decreased responsiveness of hematopoietic progenitors [22]. Mice deficient in OSM show altered regulation of G-CSF and the chemokine CXCL12 and an increase in extramedullary hematopoiesis. Also, stromal cells from OSM-deficient mice show a decreased ability to maintain in vitro hematopoiesis [23]. In embryonic hematopoiesis, OSM has been shown to be required for the expansion of multipotential progenitors from the aorta-gonads-mesonephros region [24] and in the establishment of the fetal liver microenvironment [25]. Overexpression of OSM, on the other hand, leads to increased proliferation of hematopoietic progenitors, resulting in a myeloproliferative disease [26]. Taken together, these reports show that OSM and its receptors are important both in establishment of a functional hematopoietic microenvironment and expansion of hematopoietic progenitors.

In the present paper, we wished to find out whether OSM affects expansion of human hematopoietic progenitors and repopulating HSC and whether the effects found are dependent on cross-signaling of OSM and KL. We found that OSM rescued KL-induced loss of repopulating activity and that this was associated with a transient activation of the extracellular signal-regulated kinase (ERK) pathway.

MATERIALS AND METHODS

Source of Cells

Cord blood (CB) cells were obtained from full-term newborns after informed parental consent according to guidelines approved by the ethical committee of the Technical University of Munich. Cord blood samples were collected in cord blood collection bags containing citrate buffer (Austrian Red Cross, Eugendorf, Austria, http://www.roteskreuz.at).

Enrichment and Sorting of Hematopoietic Stem Cells

Mononuclear cells were isolated from CB using density centrifugation over Ficoll ($d = 1.077$ g/ml, Biochrom AG, Berlin, http://www.biochrom.de). CB cells were magnetically enriched and subsequently sorted for expression of CD34 and CD133 and absence of CD33, CD38, and CD71 as described previously [27].

In some experiments, high-resolution cell division tracking was performed. CD133-enriched cells were labeled with 2 μM of the fluorescent dye 5-(and 6-)carboxy-fluorescein succinimidyl ester (CFSE, Molecular Probes Inc., Leiden, The Netherlands, http://probes.invitrogen.com) in Iscove's modified Dulbecco's medium (IMDM) (GIBCO, Invitrogen, Karlsruhe, Germany) and cultured overnight in 0.22-μm-filtered serum-free medium as described previously [17, 28]. After overnight culture, CFSE-labeled cells were stained with antibodies (CD34-APC, Beckman Coulter, Krefeld, Germany, http://www.beckmancoulter.com) and a mixture of phycoerythrin-conjugated antibodies against CD33, CD38, and CD71. The stained cells were then sorted for the presence of CD34 and the absence of CD33, CD38, and CD71 and into narrow gates to facilitate high-resolution cell division tracking [17].

In the Western blot and real-time polymerase chain reaction (PCR) experiments, mononuclear cells cord blood cells were enriched for the expression of CD34 using magnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com). The CD34-enriched cells were not sorted further, but otherwise treated and cultured as the cells sorted above.

Serum-Free Culture

Lin$^-$CD34$^+$ or CFSE$^+$Lin$^-$CD34$^+$ cells were resuspended in a serum-free medium (20% BIT9500 [Stem Cell Technologies, Vancouver, BC, Canada, http://www.stemcell.com], 80% IMDM [GIBCO, Invitrogen], penicillin/streptomycin [GIBCO], mercaptoethanol) at a maximum of 10^4 cells per milliliter. Cells were stimulated with KL (100 ng/ml), Flt3-ligand (FL, 100 ng/ml), IL-6, IL-11, OSM, CT-1, or CNTF (all at 50 ng/ml and all obtained from R&D Systems Inc., Minneapolis, http://www.rndsystems.com) as described in the text. The cells were cultured for 4 (CFSE experiments) to 6 days (other experiments) and harvested.

In Vitro Assays for Mature and Immature Progenitor Cells

To determine the presence of mature cells, formation of hematopoietic colonies was determined by standard criteria. Colony formation was assessed before and after serum-free culture. After 6 days, cells were harvested and 100–200 input cell equivalents were plated in growth factor-supplemented methylcellulose (H4435, Stem Cell Technologies). After 14 days, colony-forming cells were scored. Cobblestone area-forming cells (CAFC) were determined by culturing cells on 0.1% gelatin-coated 96-well plates overlayed with FBMD-1 stromal cells. Cultures were demidepopulated and replaced with fresh medium (IMDM with 20% fetal calf serum, 20 ng/ml G-CSF [Neupogen, Roche Diagnostics, Basel, Switzerland, http://www.roche-applied-science.com], and 10 ng/ml IL-3 [R&D Systems]) each week. CAFC frequency was determined by visual assessment of the presence of cobblestone areas after 2 (CAFCw2) and 6 (CAFCw6) weeks of culture.

Immature hematopoietic progenitors were determined after long-term culture on the FBMD-1 stromal cell line in the presence of TPO and FL [29]. After 6 weeks, the whole culture was harvested by trypsin detachment and assayed for the presence of colony-forming cells (CFC) [long-term culture (LTC)-derived CFC] as above.

In Vivo Repopulation Assay

Lin$^-$CD34$^+$ cells were transplanted into sublethally irradiated (3.5 Gy, low-dose settings, KD2 Mevatron, Siemens, Munich, Germany,

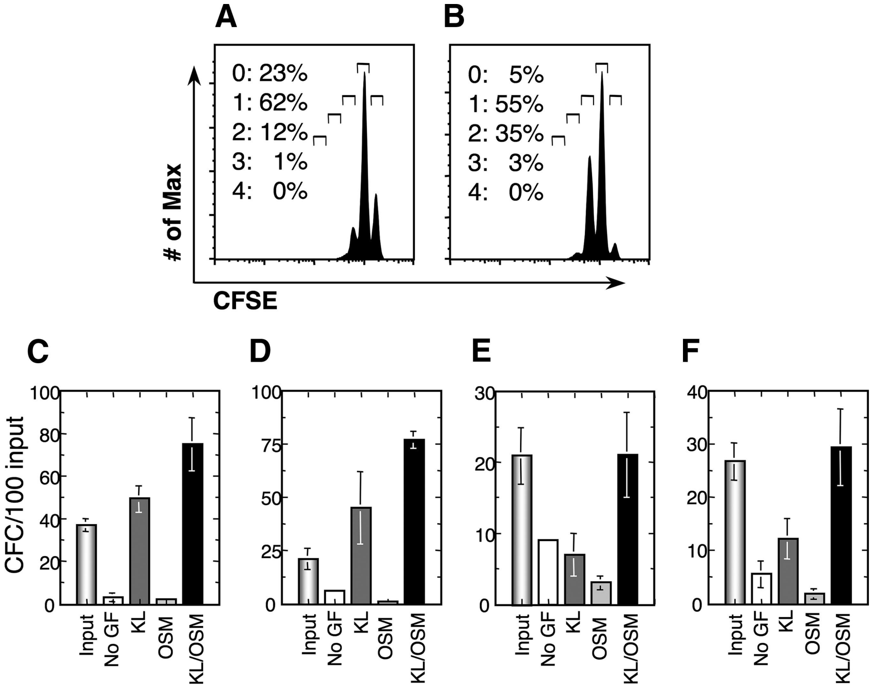

Figure 1. Oncostatin M (OSM) enhances proliferation and maintains hematopoietic progenitors. Mononuclear cord blood cells were labeled with 5-(and 6-)carboxy-fluorescein succinimidyl ester (CFSE) [17], as described in the Materials and Methods section. After sorting, the Lin$^-$CD34$^+$CD133$^+$ CFSE$^+$ sorted cells were cultured for three more days in a serum-free medium supplemented with **(A)** KIT-ligand (KL) or **(B)** a combination of KL and OSM. The two flow cytometry profiles show a representative experiment of two independent experiments. The numbers represent the percentage of the total cells in the zeroth, first, and subsequent divisions. In **(C–F)**, hematopoietic activity of cultured cells Lin$^-$CD34$^+$CD133$^+$ cord blood (CB) cells was determined after a total of 6 days in culture. Shown are the absolute numbers of **(C)** CFC, **(D)** cobblestone area-forming cells week 2 (CAFCw2), **(E)** CAFCw6, and **(F)** long-term culture-derived CFC per 100 input Lin$^-$CD34$^+$CD133$^+$ CB cells. Data shown represents mean ± SEM of five to seven independent experiments. Abbreviation: GF, growth factors.

http://www.medical.siemens.com) CB17.NOD.Prkdc$^{scid/scid}$ (NOD/SCID) mice together with 1×10^6 15 Gy-irradiated CD133$^-$ cord blood cells as carrier cells. Two days prior to irradiation, the fluid diet of the mice was changed from sterilized water to 0.2-μm-filtered water acidified with HCl to pH 5 supplemented with Neomycin (0.1 mg/ml, Sigma, Munich, Germany, http://www.sigmaaldrich.com) and polymyxin B (500 U/ml, Sigma). The mice received antibiotics throughout the entire experiment. Six to 8 weeks after transplant, the mice were sacrificed and the bone marrow, peripheral blood, and spleens were analyzed on a flow cytometer (Beckman Coulter) for the presence of mature human blood cells. For this purpose, the cells were stained for myeloid cells with a combination of CD45/CD71 and CD16/CD66b and for B-lymphoid cells using a combination of CD19/CD20 and CD34 [28]. Mice with more than 0.1% of both human myeloid and lymphoid cells in their bone marrow were considered positive. The frequency of NOD/SCID-repopulating cells was calculated using the L-Calc software package (Stem Cell Technologies).

Western Blot Analysis

Cord blood cells were enriched for the presence of CD34 using magnetic selection (Miltenyi Biotech). Purity was ≥95% in all experiments. The cells were then starved for 4 hours at 37°C after which they were stimulated with cytokines. After stimulation, the cells were put on ice and resuspended in ice-cold phosphate-buffered saline (PBS) with Na_2VO_4 (1 mM, Sigma) and washed once in cold PBS with Na_2VO_4. The cell pellet was then lysed in liquid nitrogen and stored at −80°C until further analysis. After thawing, the lysate was resuspended in protease inhibitor containing buffer consisting of benzamidin (5 mM, Fluka, Buchs, Switzerland, http://www.sigmaaldrich.com), phenylmethanesulfonyl fluoride (0.1 M, Fluka), Na_2VO_4 (0.1 M, Sigma-Aldrich), and an inhibitor cocktail (Roche). The insoluble debris was pelleted. Since the protein content of the soluble protein was usually very low, the protein was precipitated using ice-cold trichloroacetate (10%, Sigma-Aldrich). The pelleted protein was washed once in ice-cold acetone, after which it was dried. The pellet was then resuspended in SDS-containing loading buffer. For the analysis, the lysed cells were boiled and loaded onto SDS-Page under reducing conditions. After electrophoresis, the proteins were blotted onto nitrocellulose paper. The blotted nitrocellulose was then blocked and treated with different antibodies as recommended by the manufacturers. For this analysis we used the following antibodies: Akt1/pS473-v-akt murine thymoma viral oncogene homolog (AKT)1 (both from Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com), signal transducer and activator of transcription 3 (STAT3) (F-2, Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com)/pY205-STAT3 (B-7, Santa Cruz), ERK (K-23, Santa Cruz), pY204-ERK (E4, Santa Cruz), p38 (Santa Cruz), T180/Y182-p38 (Cell Signaling), p90 ribosomal S6 kinase (RSK) (RSK1/RSK2/RSK3, Cell Signaling), and FOXO3A (N-15, FKHRL1, Santa Cruz). Signals were visualized using polyclonal secondary peroxidase-labeled antibodies (Amersham Biosciences, Piscataway, NJ, http://www.amersham.com) and chemiluminescence (Supersignal West Femto, Pierce, Rockford, IL, http://www.piercenet.com). Signals were recorded on Kodak films (http://www.kodak.com). Signal intensities were determined using the ImageJ software package (NIH).

Preparation of mRNA and Real-Time PCR

Total RNA was isolated using TriZol (Invitrogen, Karlsruhe, Germany). First-strand cDNA was produced using poly-T primers (Invitrogen). For real-time PCR, 2 μl cDNA was amplified using specific primers and the SYBR Green PCR master mix (Applied Biosystems, Darmstadt, Germany, http://www.appliedbiosystems.com). All reactions were run on an ABI 7,900 sequence detector (Applied Biosystems). All primer pairs produced a minimum of primer dimers as determined by melting curve analysis. Also, dilution series showed that product amplification was similar for all primer pairs used. The primers used in this study are listed in supplemental online Table 1. To estimate relative gene representation, all expression intensities (represented by ΔC_t values) were normalized with regard to the expression of the housekeeping gene and UPF2 regulator of nonsense transcripts homolog. In our hands, using this gene as the housekeeping gene gave more stable results as the use of other more commonly used genes, including GAPDH, HPRT, and RPLP0 (not shown).

Statistics

Frequencies of CAFC and NOD/SCID-repopulating cells were determined from limiting dilution data sets using the L-Calc statistical software package (StemCell Technologies). Comparisons between means were performed using unpaired *t* tests using the InStat software (Graphpad Software, San Diego, http://graphpad.com).

Table 1. KL and KL/OSM expand CFU-GM

Day of culture	Cytokine or cytokine cocktail	Mean BFU-E	Mean CFU-GEMM	Mean CFU-GM	Mean Total CFC
0 (input)	None	10.3 ± 2.3	6.5 ± 1.2	20.5 ± 3.0	37.3 ± 3.1
6/7	No GF	1.3 ± 0.5	0.7 ± 0.6	1.3 ± 0.8	3.3 ± 2
	KL	2.8 ± 1.4	5.2 ± 1.5	41.6 ± 4.3	49.6 ± 6.2
	OSM	1.7 ± 1.0	0.3 ± 0.3	0.3 ± 0.3	2.3 ± 1.5
	KL/OSM	6.8 ± 2.6	11.4 ± 2.5	57.2 ± 9.3	75.3 ± 12.4

Cord blood-derived CD34+, CD133+, (CD33/CD38/CD71)-cells were cultured 6 days in serum-free medium with addition of different cytokines. The harvested cells were plated in growth factor-supplemented methylcellulose (H4435, StemCell Technologies) and cultured at 37C. After 14 days, the cultures were scored for presence of BFU-E, CFU-GEMM, and CFU-GM using standard criteria. The numbers represent the number of CFC per 100 input CD133$^+$CD34$^+$(CD33/38/71)$^-$ cells ± SEM of 4 independent experiments.
Abbreviations: BFU-E, erythroid burst-forming unit; CFU-GM, granulocyte, monocyte-colony-forming unit; CFU-GEMM, granulocyte, erythroid, monocyte, megakaryocyte-colony forming unit; GF, growth factors; KL, Kit-ligand; OSM, oncostatin M.

RESULTS

We compared here serum-free cultures of untreated CB CD34$^+$ cells and cells treated with either KL or OSM or both. As it has been reported that HSC activity is not maintained by either factor alone, and we previously found that early progenitors are maintained by combinations of KL and H-IL6 [19], we wished to find out whether the two factors collaborated to maintain HSC activity. For this purpose, we sorted CD34$^+$CD133$^+$, which did not express CD33, CD38, or CD71 (Lin$^-$, [27]). Sorted cells as well as cells cultured for 6 days were screened for in vitro and in vivo hematopoietic activity.

OSM Increases KL-Induced Proliferation and Maintenance of Early Progenitors

Since it has been shown that, in the murine system, the combination of KL and IL6 stimulates cell division [10], we studied proliferation using a high-resolution cell division tracking methodology [17, 27]. In a time frame of 4 days, we did not detect cell divisions in the absence of growth factors or with 50 ng/ml OSM alone (not shown). However, KL induced cell divisions such that 23% undivided cells remained (which corresponds to 40% of the input population) (Fig. 1A). Interestingly, KL and OSM collaborated to induce even more proliferation such that only 5% cells of the total cell population remained undivided (which corresponds to 14% of the input population) (Fig. 1B).

The increase in proliferation was also reflected in the number of mature hematopoietic colonies detected after 6 days of culture. Whereas, without growth factors or OSM alone the number of colonies in the cultures decreased, both KL and KL/OSM increased the number of mature colony-forming cells (CFC and CAFCw2) of up to 3.7-fold (Fig. 1C: CFC: KL: 1.4-fold, KL/OSM: 2.1-fold; Fig. 1D: CAFCw2: KL: 2.2-fold, KL/OSM: 3.7-fold). With regard to the proportion of different colony types (erythroid burst-forming unit [BFU-E], granulocyte, erythroid, monocyte, megakaryocyte-colony forming unit [CFU-GEMM], and granulocyte, monocyte-colony-forming unit [CFU-GM]), KL and KL/OSM mainly expanded CFU-GM, whereas BFU-E decreased in all conditions (Table 1). However, there was no difference in proportion of small and large colonies (data not shown). When looking at earlier progenitors (CAFCw6 (Fig. 1E) and LTC-derived CFC (Fig. 1F)), we did not observe an increase in number. Here, the number of colonies not only decreased without growth factor or OSM prestimulation but also decreased in the KL-treated cultures. Interestingly, however, the number of CAFCw6 as well as the number of LTC-derived CFC was maintained in cultures prestimulated with KL/OSM, indicating that the converging KL and OSM signals maintained early progenitors whereas each factor alone was not able to do so.

Table 2. Human engraftment in NOD/SCID mice

a	Cells per mouse	Pos/total	CRU frequency	95% Confidence interval
	0	0/3		
	100	1/7		
	300	3/13	1 in 920	1 in 690 to 1 in 1,240
	1000	6/9		
	3000	3/3		

b	Treatment	Pos/total	CRU frequency	95% Confidence interval
	No GF	0/8	<1 in 8,500	1 in 3,100 to 1 in 23,000
	KL	2/14	1 in 6,500	1 in 3,200 to 1 in 13,000
	OSM	1/8	1 in 7,500	1 in 2,800 to 1 in 20,000
	KL/OSM	5/8	1 in 1,010	1 in 640 to 1 in 1,600

Sorted CD34$^+$CD133$^+$(CD33/CD38/CD71)$^-$ cells were either transplanted directly after sorting (input cells) at different doses or cultured in the presence of the indicated growth factors for 6 days, harvested and then transplanted in sublethally (3.5 Gy) irradiated NOD/SCID mice. In (a), 0 cells means that the mice only received irradiated CD133$^-$ helper cells. The mice receiving cultured cells, received the equivalent of 1000 sorted Lin$^-$CD34$^+$ CD133$^+$ cells. Six to eight weeks after transplantation, the NOD/SCID mice were sacrificed and their bone marrow stained to detect human hematopoiesis as described in the Materials and Methods section and supplementary Figure 2. Shown is the number of positive mice per total of transplanted mice. A mouse was positive when the number of both human CD15/CD66b$^+$ and CD19$^+$ cells was higher than 0.1% of the bone marrow cells. The frequency of NOD/SCID CRU and its 95% confidence interval was determined using the L-Calc software (StemCell Technologies).
Abbreviations: CRU, repopulating units; GF, growth factors; KL, Kit-ligand; OSM, oncostatin M.

KL/OSM Maintains NOD/SCID-Repopulating Activity, but KL or OSM Alone Do Not

The in vitro studies suggest that only the combination of KL and OSM promotes maintenance of early progenitors. To find out whether these in vitro results correspond to the in vivo repopulating ability, we also transplanted serum-free cultured cells into sublethally irradiated NOD/SCID mice. Six to 8 weeks after transplantation, mice were sacrificed and the level of human engraftment was determined (supplemental online Fig. 2). Limiting dilution analysis showed that the number of NOD/SCID-repopulating cells in the input population was ≈1 in 920 Lin$^-$CD34$^+$CD133$^+$ CB cells (Table 2A). After 6 days of

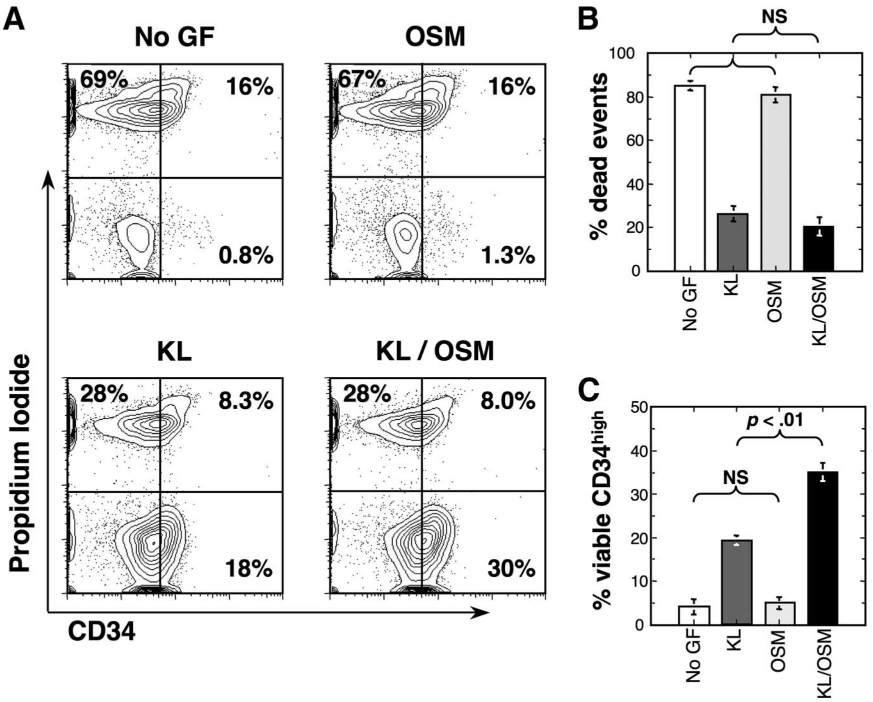

Figure 2. Survival and expression of CD34 after serum-free culture. CD133-enriched cells were sorted for the absence of lineage markers (Lin^{-}: CD3/14/15/16/19/56/Glycophorin A) as well CD33/38, and CD71, and the presence of CD34. The sorted cells were then cultured for 6 days in a serum-free medium and the indicated growth factors. After culture, the cells were stained for Lin markers (all on PE [CD3, CD14, CD15, CD16, CD19, CD56, Glycophorn A]), CD33 (on PE), CD71 (on PE), CD38 (on FITC), and CD34 (on PE-Cy5). In **(A)**, one representative experiment of three is shown and in **(B)** and **(C)** the mean results ± SEM of three independent experiments are shown. Statistical comparisons were performed using the unpaired t test. Abbreviations: GF, growth factors; KL, Kit-ligand; NS, not significantly different; OSM, oncostatin M.

culture of the equivalent of single repopulating cells (1000 Lin^{-}CD34^{+}CD133^{+} cells) without cytokines or OSM alone, repopulating activity was lost (more than a 10-fold decrease, Table 2B), suggesting that OSM not only is incapable of inducing proliferation but also is not a survival factor for repopulating cells. KL, on the other hand, does induce proliferation of Lin^{-}CD34^{+}CD133^{+} CB cells (Fig. 1A). However, transplantation of stimulated cells revealed that repopulating activity is also lost during culture with KL alone (Table 2B). This finding reflects the loss of early progenitors as measured by the CAFCw6 and LTC-derived CFC (Fig. 1E, 1F, respectively). Transplantation of the Lin^{-}CD34^{+} cells grown in the combination of KL and OSM showed a remarkable maintenance, demonstrating that OSM can counteract the loss of repopulating ability seen in cultures with KL alone (Table 2B). These results demonstrate that OSM not only amplifies KL-induced proliferation but also acts to preserve stem cell repopulating activity.

Modulation of CD34-Surface Levels by KL and OSM

From the previous results, we hypothesized that KL by itself might increase differentiation, whereas differentiation is inhibited in KL/OSM treated cultures. To investigate this, we cultured Lin^{-}CD34^{+}CD133^{+} cells as before. However, to be sure that no residual differentiation markers were present, we further depleted these cells for expression of CD3, CD14, CD15, CD16, CD19, CD56, and glycophorin A (lineage markers). Surprisingly, in all 6-day cultures, only a minor fraction of cells ($<5\%$) differentiates sufficiently to show staining with the combination of antibodies against lineage markers. In addition, one might suppose that the differences seen so far between the KL and KL/OSM cultures depend on enhanced survival. However, in both cell cultures similar levels of cell death were observed (Fig. 2A, 2B). Interestingly, we found that the fraction of cells expressing high levels of CD34 was almost twice as high in the KL/OSM-treated cells in comparison to the cells treated with KL alone (Fig. 2A, 2C).

OSM Downregulates KL-Induced ERK Activation

The above results suggest that combined signaling through KL and OSM has different effects than when signaling is induced by each cytokine alone. In particular, the combined signal preserves repopulating activity, whereas the signals propagated through each cytokine alone do not. We therefore investigated whether signaling through signal transducer and activator of transcription 3 (STAT3), AKT1 and mitogen-activated kinases (ERK) are involved in either cytokine signaling pathway and whether the combined signal alters activation of these molecules.

In CD34^{+} cells, KL and the KL/OSM combination induce similar phosphorylation patterns at 10 and 60 minutes for STAT3 and AKT (not shown). As reported by others [30], OSM as a single factor does not induce phosphorylation of either STAT3, AKT (not shown), or ERK (Fig. 3A). In contrast, KL strongly activates ERK after 10 and 60 minutes (Fig. 3A). Interestingly, in the combined KL/OSM signal, a significant decrease in ERK activation is noticeable after 60 minutes compared to that in KL alone (Fig. 3A, 3B), indicating that OSM downregulates KL-induced ERK activation. Downregulation of ERK was specific since p38 mitogen-activated protein kinase (p38 MAPK) phosphorylation was unaltered (Fig. 3A). The downregulation of ERK by the KL/OSM combination was mirrored by a decrease in activation of the ERK target p90RSK, apparent as a shift in p90 (Fig. 3A, 3B and supplemental online Fig. 2). In addition, the level of FOXO3A, which is regulated by ERK [31], was stabilized in the presence of OSM or U0126 (Fig. 3A, 3B). These biochemical results demonstrate that OSM specifically modulates KL-mediated ERK signaling by downregulating ERK and its downstream signaling events.

In parallel gene expression experiments, an increase in the differentiation-associated factor PU.1 is noted after stimulation with both cytokines as single factors, whereas in the KL/OSM combination, PU.1 is downregulated. In contrast, KL decreased the self-renewal-associated transcription factor RUNX1, suggesting that KL promotes differentiation of CD34+ cells (supplemental online Fig. 4).

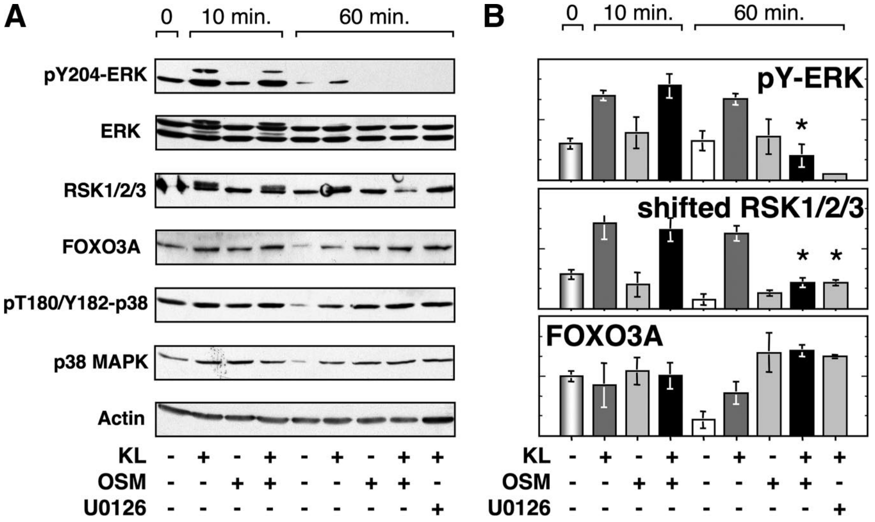

Figure 3. Oncostatin M (OSM) downregulates the extracellular signal-regulated kinase (ERK) MAPK pathway and U0126 can substitute for OSM. Western blot analysis of the ERK pathway **(A):** CD34$^+$ cord blood cells were starved 4 hour to overnight in a serum-free medium prior to addition of cytokines. An aliquot of cells was lysed each for isolation of protein and total RNA to represent the input cells ($T = 0$). Cells were simulated for the time indicated by addition of the cytokines or combinations as indicated. Each band represents the lysate of 1.2×10^6 cells. Cell lysates were blotted and incubated with the antibodies described in the Materials and Methods section. Proteins were visualized using luminescence on Kodak film. In **(B)**, the mean pixel intensity of each band was quantitated using ImageJ (NIH Software). The signal intensities were background-corrected and globally normalized for total intensity for each protein. Phospho-ERK was then determined by dividing the intensity of the lower Y204-ERK band by the intensity of total ERK plus shifted phosphoband **(A)**. Similarly, the upper (shifted) p90RSK band (for a magnification, see supplemental online Fig. 3) was divided by the intensities of the total of the two bands. FOXO3A band intensity was divided by the actin band intensity. Each column represents the mean and standard error of the mean of two to five independent experiments. Student t comparisons: $*$, $p < .05$ in a comparison of KIT-ligand (KL) with KL/OSM or KL/U0126. Abbreviations: MAPK, mitogen-activated protein kinase; RSK, ribosomal S6 kinase.

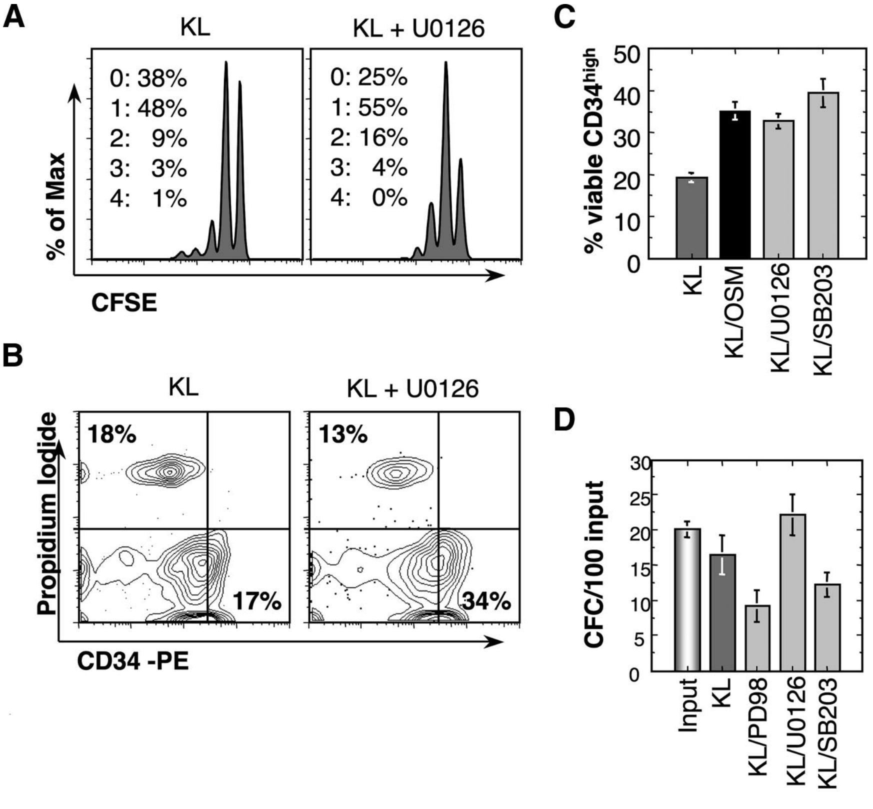

Figure 4. Effect of mitogen-activated protein kinase (MAPK) inhibitors on proliferation, survival, CD34 level, and colony-forming cells. Lin$^-$CD34$^+$CD133$^+$ cord blood cells were cultured for a total of 4 days and analyzed for **(A)** cell divisions, **(B, C)** survival, and **(C)** intensity of CD34 expression. In **(A)**, the numbers represent the percentage of the total cells in the zeroth, first, and subsequent divisions. In **(D)**, the effect of the different MAPK inhibitors on maintenance of colony-forming ability is shown. Experiments were performed as described in the Materials and Methods section and in Fig. 2. Abbreviations: CFC, colony-forming cells; KL, Kit-ligand; PE, phycoerythrin.

The MEK Inhibitor U0126 Can Substitute for OSM

The above results suggest that OSM downregulates KL-induced ERK but not p38 activation in CD34$^+$ cells. Indeed, this was confirmed by Western blots of CD34$^+$ cells stimulated with KL, followed by treatment with the MEK inhibitor U0126 (Fig. 3A, 3B). Since the Lin$^-$CD34$^+$CD133$^+$ cells represent only a minor fraction of these cells, the Western blot results do not allow direct interpretation of ERK activation in Lin$^-$CD34$^+$CD133$^+$ cells. To explore the idea that ERK activation needs to be downregulated after KL activation, we treated stringently sorted

Table 3. Inhibition of ERK counteracts KL-induced loss of repopulating cells

Treatment	Pos/total	CRU frequency	95% Confidence interval
KL	2/14	1 in 6,500	1 in 3,200 to 1 in 13,000
KL/SB203	1/6	1 in 5,500	1 in 2,000 to 1 in 15,000
KL/U0126	4/6	1 in 910	1 in 540 to 1 in 1,500

The Lin$^-$CD34$^+$ CD133$^+$ cells were cultured for 6 days and then transplanted into sublethally irradiated NOD/SCID mice. Each mouse received the equivalent of 1000 Lin$^-$ CD34$^+$ CD133$^+$ cells. After 6 weeks, the level of engraftment was determined as shown in supplementary Figure 2. Shown is the number of positive mice per total of transplanted mice. A mouse was positive when the number of both human CD15/CD66b+ and CD19+ cells was higher than 0.1% of the bone marrow cells. The frequency of NOD/SCID repopulating cells and its 95% confidence interval was determined using the L-Calc software (StemCell Technologies).
Abbreviations: CRU, competitive repopulating units; KL, Kit-ligand; OSM, oncostatin M.

cells with MEK (U0126, PD98059) and p38 MAPK (SB203580) inhibitors. We show that after 4 days of culture the KL-treated cells cycle less than KL/MAPK inhibitor-treated cultures (Fig. 4A). The ERK kinase MEK inhibitor U0126 was the most efficient in this respect (Fig. 4A). Also, flowcytometric analysis after 6 days of culture demonstrated a similar level of high CD34 expression in the KL/MEK inhibitor (both U0126 [Fig. 4B, 4C] and SB203580 [Fig. 4C] cultures as found in the KL/OSM cultures [Fig. 2C]), without a substantial additional effect on cell death (Fig. 4B). On a functional level, we found that the KL/U0126 culture, as in KL/OSM cultures, maintained late progenitors as well as KL (Fig. 4D). To look at earlier progenitors, we transplanted the equivalent of single KL-, KL/U0126-, and KL/SB203580-treated repopulating cells. These experiments showed that only the KL/U0126-treated cells, but not KL/SB203-treated cells, show significant repopulating activity 6 weeks after transplantation (Table 3). These results confirm the specific role of ERK inactivation in the maintenance of hematopoiesis-repopulating activity.

DISCUSSION

The mechanisms responsible for maintenance of HSC activity have not been adequately elucidated. Our main findings are that cord blood-derived CD34$^+$(CD33/CD38/CD71)$^-$CD133$^+$ cells are recruited into cycle by KL. This proliferation is further increased by the addition of the gp130 stimulator OSM and is paralleled by the expansion of mature hematopoietic progenitors. However, only the KL/OSM combination also maintains high CD34 expression, early progenitors, and HSC. We further show that OSM downregulates KL-induced activation of the MAPK ERK. The effects of OSM can be mimicked by the MEK inhibitor U0126, confirming the importance of downregulated ERK phosphorylation in the preservation of high CD34 expression and maintenance of NOD/SCID-repopulating cells.

We found that KL as a single factor is capable of inducing mitogenic activity as well as expanding mature progenitors. However, we also show that KL as a single factor does not promote long-term production of colony-forming cells, a finding which parallels earlier findings with murine cells [7, 32, 33]. These observations indicate that self-renewal requires additional signals to supplement those from KIT. Signals from the KIT receptor are transmitted from a number of tyrosine residues in its cytoplasmic tail to the RAF/MEK/ERK, as well as JNK and PI3K/AKT pathways [34]. These signals cooperate with other pathways induced through a number of cytokines to promote self-renewal of hematopoietic stem cells. Cytokines known to cooperate in the induction of mitogenesis together with KL are erythropoietin (EPO), G-CSF, granulocyte-macrophage colony stimulating factor (GM-CSF), IL3, and members of the IL6 family. GM-CSF by itself does not induce ERK or PI3K activation. However, upon stimulation, KIT forms a complex with the common β chain of the GM-CSF and IL-3 receptors, leading to prolonged activation of both the ERK and PI3K pathways [35]. Similarly, cooperative signaling of EPO and KL has been shown to involve both separately activated and cooperatively activated pathways in which STAT5 and JAK2 signaling were induced through EPO whereas ERK and PI3K/AKT signaling occurred in response to both cytokines [36]. Our results show that, in CD34+ CB cells, KL induces prolonged activation of both ERK and AKT1. However, in contrast to the synergistic effects of GM-CSF or EPO, OSM downregulates KL-induced ERK activation.

Cooperative signaling between the gp130-stimulating cytokines IL6, IL11, or LIF has been shown to occur in several progenitor cell types, including murine CFU-S [9, 33, 37, 38]. IL6, IL11, and LIF all increase the mitogenic activity of KL [9, 39]. Other gp130-stimulating factors, such as OSM and CNTF, appeared to counteract the synergism between IL6 or LIF and KL [9]. Deletion of gp130 leads to lethal changes in hematopoietic development [11]. In addition, mutations of different domains of gp130 have severe negative effects on hematopoiesis, which can be counteracted by additionally deleting either the IL6R or the IL11R [40]. Conversely, overexpression of the IL6/IL6R complex, which stimulates gp130 irrespective of the cytokine specific α chain, leads to myeloproliferative disease [24, 41]. Together, these findings demonstrate that gp130-mediated signals are important for hematopoietic proliferative responses and perhaps also for self-renewal.

We here show that OSM cooperates with KL not only to promote mitogenic activity but also to expand mature progenitors. More importantly, the addition of OSM preserves the repopulating activity of C34+ CB cells expanded with KL. In contrast, in the presence of KL as a single factor, repopulating activity is lost. Thus, human cells react to the KL/OSM combination similarly to the way that murine cells react to the KL/IL6 or KL/LIF combination. How the gp130 signals cooperate with those from KIT is unclear. Gp130 signaling leads to both STAT1/3/5 signals as well as to ERK signaling [38]. Interestingly, overexpression of OSM suffices to obtain a myeloproliferative disorder [24, 42]. This suggests that OSM is one of the main stimulators of myeloid proliferation in vivo. This view is underscored by findings from the Stat4−/− mouse. Here, the myeloid compartment is defective and this could be restored by treating the mice with OSM, but not IL6 [21]. Since OSM as a single factor does not stimulate proliferation but rather causes G0-G1 arrest through ubiquitination-dependent regulation of p27 and p21 [43], other factors must collaborate with OSM to stimulate myeloproliferation. In vivo, OSM is mainly expressed by hematopoietic cells [21] and not by stromal elements [44]. On the other hand, KL is expressed as membrane-bound form and/or secreted form by many cell types including the hematopoietic stromal niche. Thus, in the hyperproliferation found in the OSM transgenic mice, it is likely that OSM and KL are expressed by different cells: hematopoietic cells and stromal cells, respectively. Indeed, the finding that in the aforementioned Stat4−/− mice OSM is specifically downregulated in T-cells [21] suggests that marrow T-cells are responsible for this effect.

Our results show that OSM specifically modulates the duration of KL-induced ERK activation and its downstream targets. Activated ERK is involved in a multitude of downstream signaling pathways. How specificity is achieved through ERK has not been definitively determined. However, some ERK-associated responses are, at least in part, specified by the duration of ERK activation. It has been shown that the duration of ERK activation determines whether ERK transcription of immediate early genes MYC, FOS, and JUN is activated or repressed [45]. This dual specificity may explain why ERK is involved in such divergent processes such as proliferation and differentiation. Indeed, in neural PC12 cells, prolonged or transient ERK signaling results in either outgrowth of neurites or proliferation, respectively [46]. The studies investigating this phenomenon led to a model in which differentiation is induced by prolonged ERK activation, whereas mitogenic responses predominate when ERK is only briefly activated [46].

Our observations show that a similar model could also apply to the hematopoietic system. Indeed, ERK activation is not detected in proliferating and self-renewing cord blood cells [47]. Furthermore, prolonged ERK activation stimulates differentiation of hematopoietic progenitor cells, whereas inhibition of MEK1 blocks differentiation [48]. Our data that U0126 can substitute for OSM in most assays are in-line with the latter observation. Our data would fit a similar model as described for PC12 cells [46] in which KL induces prolonged ERK signaling with increased differentiation. When KL is combined with OSM or U0126, proliferation is promoted due to transient downregulation of ERK phosphorylation and this is associated with preservation of NOD/SCID-repopulating activity.

We show that modulation of ERK phosphorylation is a dynamic process that also affects the ERK downstream targets p90RSK and FOXO3A. We show that OSM and U0126 stabilize FOXO3A in CD34+ cord blood cells in a manner similar to that observed for 293T cells [30]. Observations that deletion of Foxo3A in mice result in increased cycling of HSC [49] would support a role for FOXO3A in the regulation of HSC cell cycle. With regard to p90RSK, there is insufficient information to speculate on its effects on HSC. P90RSK, does, however, regulate hematopoiesis, as has been shown by experiments in which constitutively active p90RSK promoted hematopoietic transformation [50]. Whether p90RSK affects proliferation or differentiation of HSC remains to be investigated.

We also find that transient modulation of the ERK pathway modulates the expression levels of different transcription factors necessary for maintenance of stem cells: PU.1 (SPI1), RUNX1, SCL, and IRF8 [51]. The model above suggests that prolonged ERK signaling eventually leads to upregulation of differentiation-associated genes, whereas transient ERK activation might downregulate such genes. This hypothesis is true in murine progenitors where prolonged ERK activation upregulates PU.1, and MEK inhibition leads to downregulation of PU.1 [48]. Our experiments also support this hypothesis. We found that the combination of KL/OSM promotes proliferation with concomitant downregulation of PU.1. At the same time, the expression level of RUNX1, which is associated with self-renewal, remained unaltered. However, the process of differentiation in human primary cells underlies different kinetics, as we found that PU.1 downregulation is, as in ERK activation, transient. Also, we observed a decrease in absolute expression of surface CD34 in cultures stimulated with KL as a single factor, and higher CD34 levels in the KL/OSM as well as KL/U0126-stimulated cells. This could mean that downregulation of CD34 levels is one of the earliest detectable effects of stem cell differentiation. In support of this view that maintenance of HSC activity is associated with higher CD34 levels, other investigators have recently found that a decrease in PU.1 may correspond to higher CD34 protein levels and higher repopulating activity [52].

In conclusion, we demonstrate that oncostatin M cooperates with KL to increase proliferation of $(CD33/38/71)^{-}CD34^{+}$ $CD133^{+}$ cord blood cells. This increase in proliferation is associated with preservation of NOD/SCID-repopulating activity. At the molecular level, this effect is explained by the observation that OSM blunts the KL-activated ERK pathway, resulting in p90RSK activation, FOXO3A stabilization, and transient downregulation of the differentiation-associated transcription factor PU.1. We propose that maintenance of repopulating hematopoietic stem cells depends on the dynamic modulation of the ERK signaling pathway. Our findings that small molecule inhibitors of ERK signaling positively affect stem cell maintenance may lead to new strategies to improve the outcome of stem cell expansion.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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