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Stem cell factor induces Hif-1α at normoxia in hematopoietic cells

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ABSTRACT

Signaling by the receptor for stem cell factor (SCF), c-Kit, is of major importance for hematopoiesis, melanogenesis and reproduction, and the biological responses are commonly proliferation and cell survival. Thus, constitutive activation due to c-Kit mutations is involved in the pathogenesis of several forms of cancer, e.g. leukemias, gastrointestinal stromal tumors and testicular tumors. Tumor survival requires oxygen supply through induced neovascularization, a process largely mediated by the vascular endothelial growth factor (VEGF), a prominent target of the transcription factors hypoxia-inducible factor-1 (HIF-1) and HIF-2. Using Affymetrix microarrays we have identified genes that are upregulated following SCF stimulation. Interestingly, many of the genes induced were found to be related to a hypoxic response. These findings were corroborated by our observation that SCF stimulation of the hematopoietic cell lines M-07e induces HIF-1α and HIF-2α protein accumulation at normoxia. In addition, SCF-induced HIF-1α was transcriptionally active, and transcribed HIF-1 target genes such as *VEGF, BNIP3, GLUT1* and *DEC1*, an effect that could be reversed by siRNA against HIF-1α. We also show that SCF-induced accumulation of HIF-1α is dependent on both the PI-3-kinase and Ras/MEK/Erk pathways. Our data suggest a novel mechanism of SCF/c-Kit signaling in angiogenesis and tumor progression.

c-Kit is a receptor tyrosine kinase that normally controls the function of primitive hematopoietic cells, germ cells and melanocytes. Binding of stem cell factor (SCF) to c-Kit causes receptor dimerization and activation of the intrinsic tyrosine kinase activity, leading to phosphorylation of the receptor subunits as well as of downstream signal transduction molecules. Uncontrolled activity of c-Kit contributes to the formation of a variety of human malignancies, including acute myeloid leukemia and lymphoma [1].

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor composed of HIF-1α and HIF-1β/ARNT subunits. The β-subunit is constitutively expressed, whereas the stability and activity of the α-subunit are inducible. The availability of the HIF-1α subunit is primarily regulated by cellular oxygen levels [2], but also by growth factors [3]. Furthermore, survival strategies of cells under hypoxic conditions reflect induction of HIF-1-regulated genes. The promoter or enhancer regions of these genes generally contain a hypoxia-responsive element (HRE), the sequence recognized by HIF-1 [4]. Overexpression of HIF-1α has been observed in many human cancers [5], indicating that tumors take advantage of the physiological survival and proliferative response of increased HIF-1 transcriptional activity.

The known mechanisms that regulate the HIF-1α protein are O_2-dependent post-translational modifications such as prolyl hydroxylation [6]. This hydroxylation is performed by HIF-1 prolyl hydroxylases (PHDs) and results in recognition and binding of the von Hippel-Lindau (VHL) multiprotein E3 ligase complex that after binding to HIF-1α targets it for degradation by the ubiquitin/proteasomal pathway [7]. Hydroxylation of HIF-1α is inhibited at hypoxia, leading to a stabilized and active transcription factor. It appears that the mechanisms that regulate HIF-1α activity under normoxic and hypoxic conditions differ. Oxygen-dependent HIF-1 regulation principally affects HIF-1α protein stability and access to transcriptional co-activators. Normoxic growth factor-induced HIF-1α expression involves additional mechanisms, such as increased translation, leading to higher HIF-1α protein levels. HIF-2α is structurally related to HIF-1α and also upregulated under hypoxic conditions. Although several target genes are identical, they have distinctive biological functions and expression patterns [8].

Angiogenesis is a critical event in tumor progression that facilitates supply of oxygen and nutrients to the tumor, and also dissemination of cancer cells. The most important angiogenic factor is vascular endothelial growth factor (VEGF), which is transcriptionally regulated by HIF-1. Furthermore, involvement of SCF in regulation of angiogenesis has been suggested and SCF-stimulation of c-Kit expressing small cell lung cancer cells increases VEGF secretion [9,10]. Thus, knowledge about HIF-1 activation mecha-

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nisms, at hypoxia as well as at normoxia, is of major importance to understand the processes behind cancer progression and vascular disease.

Here we report that SCF can induce HIF-1α and HIF-2α protein levels in normoxic hematopoietic cells. We also demonstrate that the increase in HIF-1α protein subsequently is followed by an augmentation of HIF-1 transcriptional activity, including upregulation of HIF-1 target genes such as *VEGF, BNIP3* and *DEC1*. Furthermore, we present results showing that SCF induces HIF-1α by PI-3-kinase- and Ras/MEK/Erk-dependent mechanisms.

Materials and methods

Cell culture. M-07e cells, Kasumi-1 and Ba/F3 cells were purchased from DSMZ (Braunschweig, Germany). M-07e and Ba/F3 cells were cultured in RPMI-1640 medium (Gibco, Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated FBS (Gibco), 100 U/ml penicillin and100 μg/ml streptomycin. In the case of M-07e 10 ng/ml recombinant human interleukin-3 (IL-3) (Biosource), and in the case of Ba/F3 10 ng/ml recombinant murine IL-3 (Prospec Tany, Rehovot, Israel) was added to the medium, respectively. Kasumi-1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were starved for 6 h without growth factors in the presence of 0.5% serum, before stimulation with 100 ng/ml SCF.

Inhibitors. The MEK inhibitor U0126 was obtained from Promega (Madison, WI), the Src inhibitor SU6656 and the p38 inhibitor SB203580 were from Calbiochem (San Diego, CA). Cycloheximide and the PI-3-kinase inhibitor LY294002 were purchased from Sigma-Aldrich (St. Louis, MI).

Microarray sample preparation and data analysis. Ba/F3 cells, stably transfected with c-Kit, were starved and stimulated with SCF for 0 or 8 h. Trizol reagent (Invitrogen) and RNeasy kit (Qiagen, Hilden, Germany) were used according to manufacturer's instructions to isolate RNA. Microarray experiments were performed using Affymetrix gene chip technology. Samples were processed into labeled cRNA, hybridized onto the murine array MOE430 2.0 and detected by fluorescent scanning. Arrays were normalized, and comparisons between gene expression levels were performed by dChip software, using the perfect match/mismatch (PM/MM) analysis method. Genes were considered to be upregulated when SCF-treated versus untreated cells showed a minimum twofold induction.

Total cellular protein extraction and Western blotting. Cells were stimulated with SCF for the indicated periods of time, at 37 °C, and thereafter lysed in RIPA-buffer and processed for Western blotting as described [11]. The following primary antibodies were used: anti-HIF-1α (Abcam), anti-HIF-2α (Abcam), anti-β-actin (Sigma), and anti-phosphorylated Erk 1/2 (Santa Cruz Biotechnology). The rabbit c-Kit antibody Kit-C1, was made in-house [12].

Pulse-chase analysis. M-07e cells were starved, stimulated with SCF for 0 or 24 h, or 100 μM cobalt chloride ($CoCl_2$) for 18 h and then washed and incubated in methionine/cysteine-free DMEM (Gibco) supplemented with 0.5% heat-inactivated-FBS, 100 U/ml penicillin, 100 U/ml streptomycin, 4 mM L-glutamine, 10 mM HEPES and 50 μCi/ml of [^{35}S] methionine/cysteine Promix (Amersham Biosciences, Buckinghamshire, UK) for 15 min, chased with cold methionine and cysteine, lysed and immunoprecipitated essentially as described using antibodies against HIF-1α [13].

Quantitative real-time PCR (QPCR). RNA extraction, cDNA synthesis, and Q-PCR reactions with SYBR Green PCR master mix (Applied Biosystems) were performed as described [14]. Expression levels of genes of interest were normalized to the expression of three housekeeping genes (*SDHA*, *YWHAZ*, and *UBC*) not affected by reduced oxygen. Primers were designed using Primer Express (Applied), and sequences are given as Supplementary Table 1.

Transfections, luciferase assay and small inhibitory RNA (siRNA) treatment. For luciferase assays, triplicates of 8×10^4 SK-N-BE(2)c neuroblastoma cells were seeded in 24-well chamber plates (Corning, Corning NY) one day before transfection. This cell line was chosen due to its high transfection efficiency and its lack of normoxic c-Kit protein expression. Subsequently, cells were transfected with a vector containing three hypoxia-response elements (HREs) coupled in tandem to a luciferase reporter gene, c-Kit cDNA expressing plasmid, and a TK-*Renilla* luciferase vector. Transfected cells were stimulated with SCF for 24 h. Detection and normalization of luciferase activity (HRE-Luc/TK-Renilla Luc) were then performed by employing the Dual Luciferase Reporter Assay System (Promega). In a set of experiments, $2–3 \times 10^6$ M-07e cells were transfected with 2.5 μg of HPLC-purified annealed siRNA duplexes (Ambion, Austin, TX) directed against *HIF-1α* mRNA (siHIF-1), or control (siSCR) using the Amaxa Nucleofector Device (Amaxa Biosystems, Cologne, Germany). Cells were recovered overnight before starvation, followed by SCF stimulation, and then harvested for QPCR analysis or Western blotting.

Flow Cytometry. M-07e cells were labeled with PE-anti-human CD117, for c-Kit detection, or PE-mouse IgG1κ antibodies (as isotype control). Antibodies were purchased from Biolegend. Flow cytometry was performed with a FACS sort instrument (BD Biosciences San Jose CA).

Results

SCF induces HIF-1α and HIF-2α accumulation in c-Kit expressing hematopoietic cells

Using microarray technology we found that SCF stimulation of the murine hematopoietic pro-B cell line Ba/F3, stably transfected with c-Kit, upregulated several genes known to be induced by the transcription factor HIF-1 at hypoxia (reviewed in [15]; Supplementary Table 2). We therefore investigated the effects of SCF on HIF-1α protein expression, accumulation and activity at normoxia. The human acute megakaryoblastic leukemia cell line M-07e, which expresses c-Kit endogenously was treated with SCF, or placed in a hypoxic environment (1% O_2) as positive control. As seen, HIF-1α protein accumulation was induced by SCF in M-07e cells (Fig. 1A). Similar data were obtained using the pro B-cell line Ba/F3 transfected with c-Kit (data not shown). The AML cell line Kasumi-1, carrying an activating mutation of c-Kit (N822K) that renders it independent of external factors for growth, was stimulated with SCF and analyzed for HIF-1α expression. Even in the absence of SCF, expression of HIF-1α could be detected, which was further increased by ligand stimulation (Fig. 1B). Since the related protein HIF-2α is regulated by hypoxia, we investigated whether c-Kit could also induce HIF-2α protein expression. SCF-stimulation of M-07e cells was found to increase HIF-2α protein expression (Fig. 1C), with similar kinetics compared to HIF-1α. Thus, induction of both HIF-1α and HIF-2α occurs following SCF stimulation, but with a somewhat delayed kinetics compared to hypoxic stimulation. To characterize the mechanisms involved in HIF-1α accumulation after activation of c-Kit signaling pathways, we evaluated the mRNA levels of HIF-1α in M-07e cells stimulated with SCF. The mRNA levels of HIF-1α were not significantly affected by SCF stimulation indicating that the increased HIF-1α protein levels were not due to enhanced mRNA transcription, but rather to later events (Fig. 1D). We further analyzed the HIF-2α mRNA levels in SCF-stimulated M-07e cells and found, contrary to the HIF-1α mRNA levels, an upregulation of HIF-2α mRNA levels (Fig. 1D).

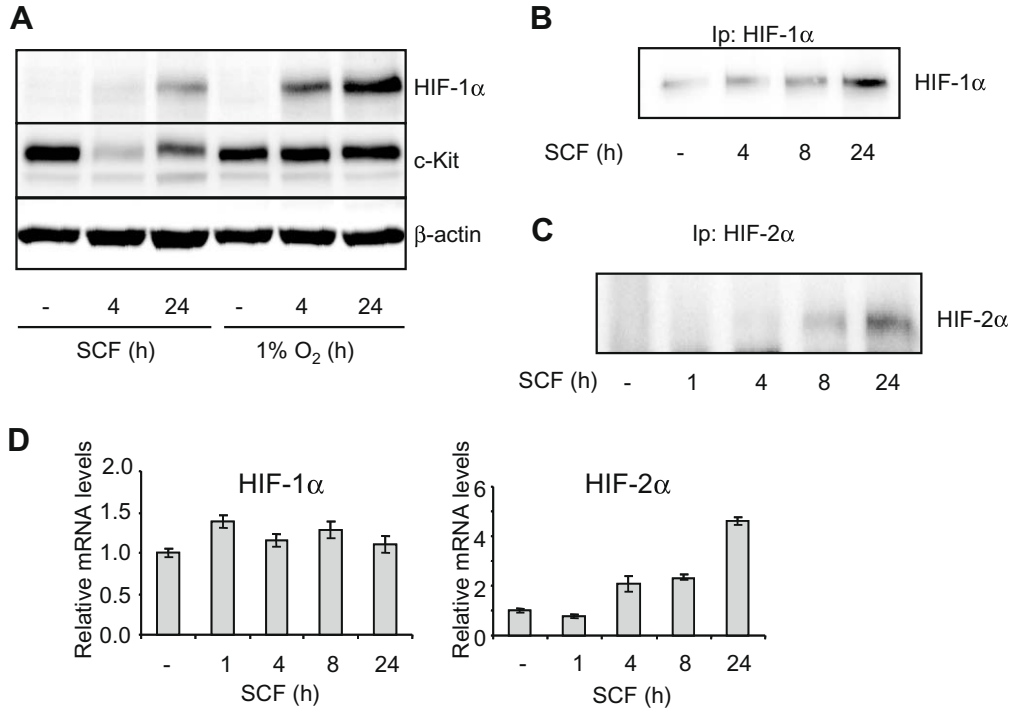

Fig. 1. SCF stimulation of hematopoietic cells induces HIF-1α and HIF-2α protein expression under normoxic conditions. (A) M-07e cells were starved before SCF-stimulation for 0, 4 or 24 h, respectively. M-07e cells cultured at hypoxia were used as control. Whole cell lysates were prepared and analyzed by Western blotting using antibodies against HIF-1α, c-Kit and β-actin. (B) Kasumi-1 cells were starved of serum followed by stimulation with SCF for 0, 4, 8 and 24 h. Cells were lysed and immunoprecipitated with an antibody against HIF-1α, separated by SDS–PAGE, and probed with an antibody against HIF-1α. (C) M-07e cells were starved and stimulated with SCF for 0, 1, 4, 8 and 24 h. Cell lysates were investigated for HIF-2α protein levels by immunoprecipitation using an antibody against HIF-2α, followed by Western blotting. (D) M-07e cells were starved and stimulated with SCF for the indicated periods of time followed by RNA isolation and cDNA synthesis. QPCR analysis was then performed using specific primers for HIF-1α or HIF-2α.

Thus, in contrast to HIF-1α, HIF-2α seems to be regulated by c-Kit activation at the mRNA level.

SCF signaling enhances transcriptional activity of HIF-1

Next we investigated if the increased HIF-1α protein levels after SCF stimulation of M-07e cells also resulted in increased HIF-1 transactivation. We therefore performed an HRE-driven luciferase reporter gene assay. Here we used the SK-N-BE(2)c cell line that lacks endogenous c-Kit expression (data not shown). Cells were stimulated with SCF for 24 h followed by detection and normalization of luciferase activity. From this experiment we concluded that the SCF-induced HIF-1α was functional and could activate transcription via a general HRE (Fig. 2A).

SCF stimulation leads to increased expression of HIF-1 target genes

Several of the SCF-induced genes from the microarray experiment described above are genes known to be upregulated at hypoxia under control of HIF-1α. To confirm these results, we investigated by QPCR the mRNA levels of a subset of genes in SCF-stimulated M-07e cells. We found an induction of the HIF-1-regulated genes *DEC1, VEGF, BNIP3* and *GLUT1* [15] in response to SCF stimulation (Fig. 2B). To examine if these changes in gene expression were dependent on HIF-1α transcriptional activity, we used small inhibitory RNA (siRNA) to knock down the expression HIF-1. The reduced HIF-1α levels also resulted in a decrease in mRNA levels of *DEC1*, *VEGF*, *BNIP3* and *GLUT1* (Supplementary Fig. 1A). Confirmation of both HIF-1α mRNA and protein levels after cognate siRNA treatment showed a reduction to approximately 58% of original levels (Supplementary Fig. 2A and B).

The effect of SCF treatment on the synthesis and stability of HIF-1α protein in M-07e cells

To investigate HIF-1α degradation we analyzed HIF-1α protein stability by [^{35}S] methionine/cysteine pulse-chase analysis. Newly synthesized HIF-1α expression was declined after 15 min and almost lost after 60 min (Fig. 3A). Under normoxic conditions without any addition of cytokines, HIF-1α protein half-life has been measure to be about 5 min [16]. As control, HIF-1α was stabilized by use of cobalt chloride. Next, we wanted to examine the SCF effect on HIF-1α synthesis using the protein translation inhibitor cycloheximide (chx). The addition of cycloheximide led to diminished HIF-1α protein expression, indicating that the HIF-1α accumulation seen is dependent on ongoing protein synthesis (Fig. 3B). We therefore propose that the increase levels of HIF-1α protein after treatment with SCF is a combination of both increased stability and an increased protein synthesis.

Induction of HIF-1α protein expression by SCF is dependent on PI-3-kinase activity and the Ras/MEK/Erk pathway

Under hypoxic conditions the increase in HIF-1α protein is a result of increased stability and inhibited degradation. In contrast, it has been reported that in the case of growth factor stimulation at normoxia, increased levels of HIF-1α are mainly due to increased *HIF-1α* mRNA translation via a PI-3-kinase-dependent pathway [3]. The PI-3-kinase pathway is also one of the major pathways activated in SCF/c-Kit signaling [17]. Therefore we investigated if activation of PI-3-kinase was needed for the increased levels of HIF-1α protein after stimulation with SCF. Using Western blotting we could detect a remarkable decrease in HIF-1α protein

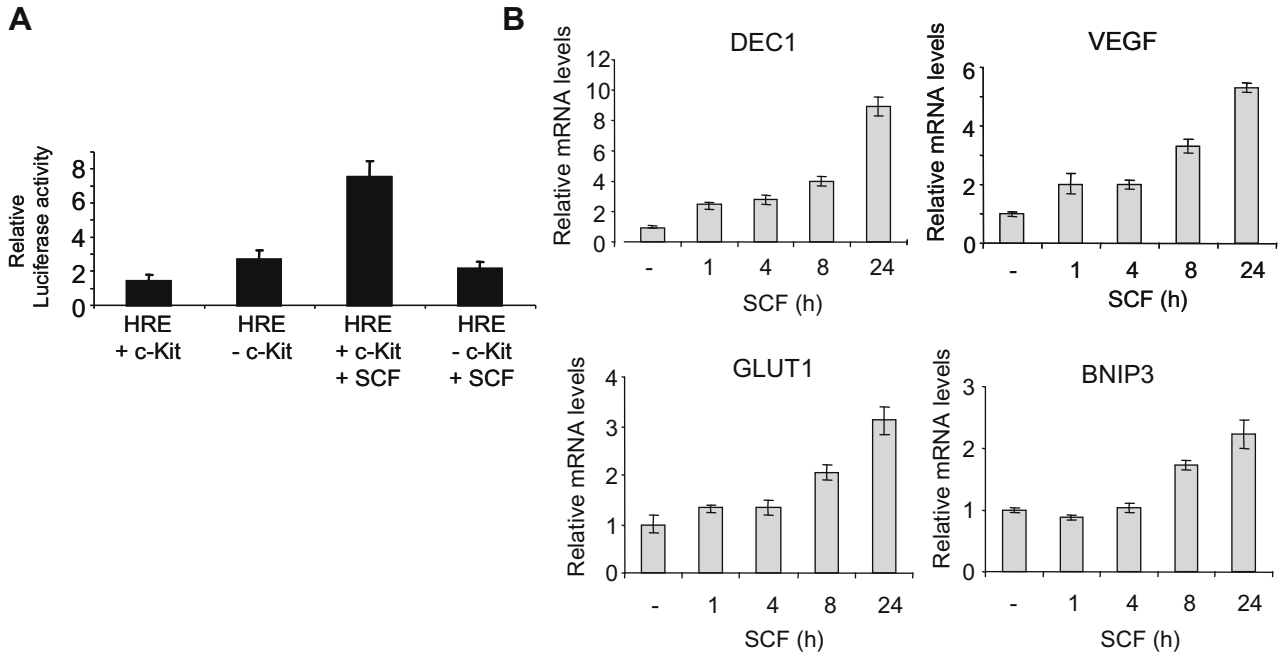

Fig. 2. SCF increases HIF-1α-mediated HRE activity and upregulates known HIF-1 target genes. (A) SK-N-BE(2)c neuroblastoma cells were transfected with a vector containing three HREs coupled to a luciferase reporter gene, together with a c-Kit cDNA expressing plasmid, and a TK-*Renilla* luciferase vector for determination of transfection efficiency. Transfected cells were stimulated with SCF for 24 h, followed by harvesting and determination of luciferase activity. (B) M-07e cells were starved before SCF stimulation as indicated, followed by RNA isolation and cDNA synthesis. QPCR analysis was then performed using specific primers for *DEC1*, *VEGF*, *GLUT1* and *BNIP3*.

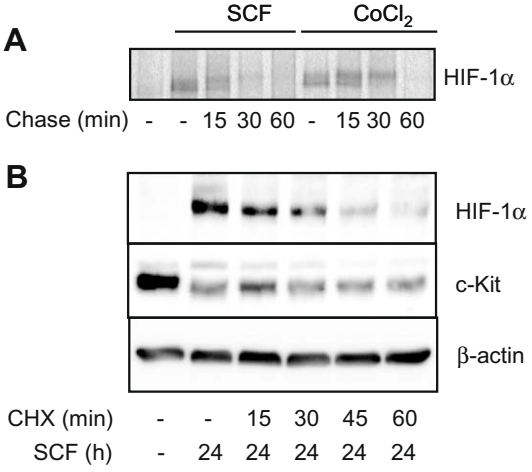

Fig. 3. SCF-induced HIF-1α accumulation occurs through a posttranscriptional mechanism. (A) M-07e cells were starved and stimulated with SCF for 24 h or cobalt chloride for 18 h, followed by pulse-labeling with [^{35}S] methionine/cysteine for 15 min and thereafter chased with unlabeled complete medium with methionine and cysteine in excess for 15, 30, 45, and 60 min. Cell lysates were subjected to immunoprecipitation using a HIF-1α antibody. (B) Western blot analysis of HIF-1α and c-Kit proteins from starved M-07e cells, subsequently stimulated with SCF for 24 h. Cycloheximide (CHX, 10 μg/ml) was added the last 60, 45, 30 or 15 min before harvesting.

expression in SCF-stimulated cells pretreated with LY294002, compared to cells not pretreated with the inhibitor (Fig. 4A).

To further investigate which c-Kit signaling pathway(s) mediate the stabilization of HIF-1α we used inhibitors of known effectors of c-Kit, such as Src and MEK. Src is known to be necessary for c-Kit mediated activation of the Ras/MEK/Erk pathway [12]. Furthermore, c-Kit mediated activation of PI-3-kinase involves in part Src-dependent phosphorylation of the scaffolding protein Gab2 [18]. M-07e cells were pretreated with SU6656 or U0126 before stimulation with SCF. Both inhibitors suppressed induction of HIF-1α protein expression almost completely. As a negative control we investigated the effect of the p38 inhibitor SB203580 on HIF-1α accumulation after SCF treatment. SB203580 did not affect the protein levels of HIF-1α compared to cells not pretreated with this inhibitor (Fig. 4C). These data indicate that the HIF-1α protein accumulation detected after SCF stimulation is a cause of multiple, possibly interacting signaling pathways, or by different regulating events that together contribute to increased HIF-1α protein levels. Incubation of cells with inhibitors without SCF stimulation did not alter the expression of c-Kit protein (Supplementary Fig. 3A–C).

Discussion

It has previously been reported that SCF, together with the hypoxia-mimicking agent $CoCl_2$, induces an increase in HIF-1α levels in small-cell lung cancer cells, relative to cells treated with $CoCl_2$ alone. In the same study, enhanced *VEGF* gene transcription was demonstrated after SCF stimulation [10]. The results in our study present evidence that SCF-induced c-Kit signaling alone can substantially increase levels of HIF-1α protein under normoxic conditions in three different hematopoietic cell lines, with a subsequent increase in gene transcription of several HIF-1 driven genes such as *DEC1, GLUT1, BNIP3* and *VEGF*.

Several different types of cancer and leukemia show abnormal SCF/c-Kit expression and/or activation, e.g. hematopoietic malignancies, tumors of the gastrointestinal system, and testicular cancer [1]. Angiogenesis is an important strategy for survival and metastasis of tumor cells, and we can propose a putative role of SCF-induced HIF-1α and VEGF in tumor revascularization.

Several reports over the recent years show involvement of transforming oncogenes such as *Src*, *Ras* and *Akt* in HIF-1α stabilization under normoxic conditions, an effect possibly reinforced by inactivation or loss of tumor suppressor genes. In addition, overexpression of HIF-1α is frequently seen in many human can-

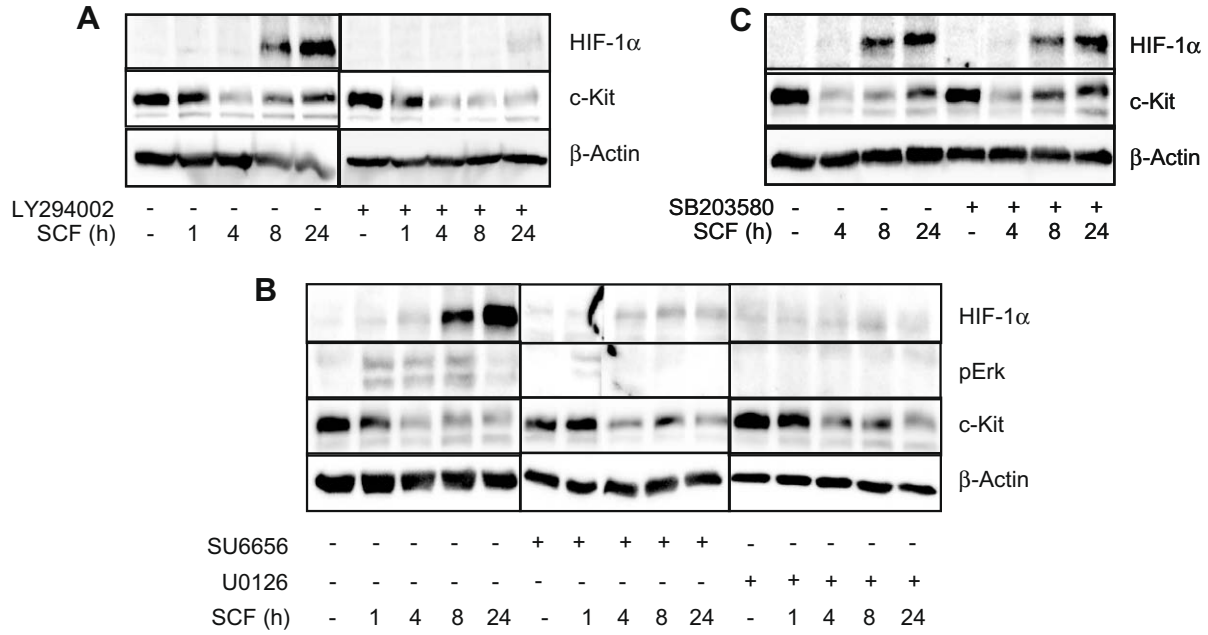

Fig. 4. SCF-induced HIF-1α stabilization is dependent on several signal transduction pathways downstream of c-Kit. M-07e cells were starved and thereafter preincubated with (A) the P13-kinase inhibitor LY294002 (10 μM), (B) the Src family kinase inhibitor SU6656 (2 μM), the MEK inhibitor U0126 (10 μM or (C) the p38 inhibitor SB203580 (10 μM) for 30 min, and subsequently stimulated with SCF for indicated periods of time. Whole cell lysates were investigated by Western blotting using antibodies against HIF-1α pErk, β-actin or c-Kit.

cers [5]. The induction of HIF-1α by growth factors at normoxia is suggested to be a result of increased mRNA translation and/or gene transcription, although some reports point towards increased stabilization and inhibited degradation of the protein. Our data suggest that the mechanism behind SCF induced HIF-1α accumulation is the result of both increased protein translation and decreased degradation (Fig. 3A and B).

Activation of c-Kit elicits two major signaling pathways, the PI-3-kinase/Akt and Ras/MEK/Erk pathways. Our data show that activation of both these pathways is necessary to achieve high levels of HIF-1α protein expression in SCF-stimulated M-07e cells under normoxic conditions (see Fig. 4A and B). Similarly, it was found that HIF-1α induction following IGF-I stimulation was dependent on Erk, while hypoxia driven HIF-1α expression was independent of Erk [19]. Also investigating growth factor induced HIF-1α induction, Treins et al. demonstrated that IGF-I stimulation of human retinal epithelial cells led to upregulation of HIF-1α expression in a PI3-kinase/mTOR and Erk-dependent manner by a posttranscriptional mechanism [20].

Our data suggest a positive regulation by SCF of HIF-1α expression at late time points. After SCF stimulation, we observed an increase in HIF-1α levels and activity. This could have several implications, including stimulation of VEGF-mediated angiogenesis and tumor metastasis. Recent findings suggest that VEGF can induce upregulation of c-Kit expression in adipose tissue derived stem cells [21]. We found that the AML cell line Kasumi-1, carrying an activating mutation of c-Kit (N822K [22]) showed elevated levels of HIF-1α even in the absence of ligand stimulation at normoxia (Fig. 1C) indicating a possible role in leukemic angiogenesis [23].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.09.102.

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