

Signal Transducer and Activator of Transcription 3 Is Required for Hypoxia-Inducible Factor-1 α RNA Expression in Both Tumor Cells and Tumor-Associated Myeloid Cells

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Abstract

Hypoxia-inducible factor 1 (HIF-1) is a potent tumorigenic factor. Its α subunit (HIF-1 α), which is tightly regulated in normal tissues, is elevated in tumors due to hypoxia and overactive growth signaling pathways. Although much is known about HIF-1 α regulation in cancer cells, crucial molecular targets that affect HIF-1 α levels modulated by both hypoxia and oncogenic signaling pathways remain to be identified. Additionally, whether and how the tumor microenvironment contributes to HIF-1 α accumulation is unclear. This study shows a novel mechanism by which HIF-1 α availability is regulated in both cancer cells and in myeloid cells in the tumor microenvironment. We show a requirement of signal transducer and activator of transcription 3 (Stat3) for HIF-1 α RNA expression under both hypoxia and growth signaling conditions. Furthermore, tumor-derived myeloid cells express elevated levels of HIF-1 α mRNA relative to their counterparts from normal tissues in a Stat3-dependent manner. Additionally, Stat3 activity in the nontransformed cells in the tumor milieu affects HIF-1 α RNA expression of the entire growing tumor. Consistent with a role of Stat3 in regulating HIF-1 α RNA transcription, elevated Stat3 activity increases HIF-1 α promoter activity, and Stat3 protein binds to the HIF-1 α promoter in both transformed cells and in growing tumors. Taken together, these findings show a novel mode by which HIF-1 α is regulated not only

in cancer cells but also in the tumor-associated inflammatory cells, suggesting Stat3 as an important molecular target for inhibiting the oncogenic potential of HIF-1 induced by both hypoxia and overactive growth signaling pathways prevalent in cancer. (Mol Cancer Res 2008;6(7):1099–105)

Introduction

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor composed of an α (HIF-1 α) and a β subunit (HIF-1 β) subunit. HIF-1 plays a critical role in tumor angiogenesis, metastasis, growth, and resistance to therapies (1-3). Under physiologic oxygen conditions and without additional stimulation by growth/oncogenic signals, HIF-1 α levels in cultured cells are very low and usually undetectable. This is due to its continual and rapid destruction via proteosomal degradation in the presence of O₂. Degradation of HIF-1 α during normoxia is extremely rapid, with HIF-1 α half-life estimated to be less than 5 minutes. As the level of O₂ decreases, which occurs in tumors, proteosomal degradation ceases and HIF-1 α protein accumulates (1, 4). In addition, during normoxia, growth signaling also affects HIF-1 α syntheses. It has been well established that activation of many oncoproteins and signaling pathways up-regulates HIF-1 α protein synthesis and HIF-1 transcriptional activity, contributing to tumor angiogenesis, invasion, and progression (1). Known stimulators of HIF-1 α protein synthesis include Src, epidermal growth factor receptor, human epidermal growth factor receptor 2, and interleukin-6 receptor. These stimulators of HIF-1 α signal through phosphatidylinositol 3-kinase and Akt, leading to an increased rate of HIF-1 α protein synthesis (1, 3).

Signal transducer and activator of transcription 3 (Stat3) is a point of convergence for oncogenic growth signaling pathways such as Src, epidermal growth factor receptor, human epidermal growth factor receptor 2, interleukin-6 receptor, and c-Met (5-8), and is frequently activated in cancers of diverse origins (8). Stat3 has been shown to play a critical role in promoting cancer cell survival, proliferation, tumor angiogenesis, metastasis, and tumor immune evasion/suppression (7-11). Recently, Stat3 has also been shown to be required for HIF-1 α protein expression induced by these oncoproteins/growth signaling

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Note: G. Niu, J. Briggs, and J. Deng contributed equally to this work.

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molecules (12). Furthermore, hypoxia has been shown to activate Stat3 in a renal cell carcinoma cell line, contributing to both stability and synthesis of HIF-1 α protein (13). The precise underlying molecular mechanism(s) by which Stat3 contributes to the multilevel HIF-1 α regulation, however, has not been defined. Although a critical role of the tumor microenvironment, which include many myeloid cells, in tumorigenesis and resistance to therapy is becoming increasingly recognized, whether they contribute to HIF-1 availability in tumors and how HIF-1 might be regulated in the tumor microenvironment remain largely unknown.

In this study, we show that Stat3 is required for hypoxia-induced HIF-1 α steady-state levels in both v-Src-transformed mouse 3T3 cells and in a human melanoma cell line with constitutively activated c-Src. Importantly, expression of HIF-1 α mRNA in both transformed cells and the tumor stromal inflammatory cells is significantly higher than their counterparts in normal tissues. In addition, Src and Stat3 activity synergize to enhance transcriptional activation of the HIF-1 α promoter. Stat3 protein binds to the HIF-1 α promoter both in tumor cells and in the entire growing tumor. Although it is well established that the HIF-1 α steady-state level is primarily regulated by protein stability (determined by O₂ concentration) and protein synthesis (growth signaling), our data show that blocking a key transcription factor (Stat3) can effectively inhibit HIF-1 activity before other regulatory steps. These results further indicate that targeting Stat3 in both tumor cells and the tumor stroma can inhibit HIF-1 oncogenic potential at multiple levels.

Results

Stat3 Is Necessary for HIF-1 α Induction by Hypoxia in Transformed Cells with Elevated Src Activity

Hypoxia is known to cause endogenous c-Src kinase activation (14). Expression of the activated v-Src oncoprotein leads to elevated levels of HIF-1 α under both normoxic and hypoxic conditions (15-17). In addition, a recent study indicated that Stat3, similar to Src, can be activated by hypoxia (13). However, whether Stat3 is necessary for hypoxia-induced, Src-mediated HIF-1 α up-regulation has not been shown. Our initial experiments involving HIF-1 α induction by hypoxia used BALB/c-3T3 fibroblasts transformed by the activated v-Src oncoprotein. The 3T3/v-Src cells have elevated HIF-1 α expression and high levels of Stat3 DNA-binding activity (Fig. 1A). Treating these cells with hypoxic mimic (CoCl₂) increased the steady-state level of HIF-1 α in both 3T3/v-Src cells with or without an empty control retroviral vector (MSCV; Fig. 1A). By contrast, 3T3/v-Src cells transduced with a retroviral vector encoding dominant-negative Stat3 (Stat3D) resulted in dramatic reduction in Stat3 DNA-binding activity and HIF-1 α steady-state levels under both normoxia and hypoxia conditions (Fig. 1A). Similarly, when these cells were subjected to 1% O₂ (hypoxia), HIF-1 α expression was strongly induced in the MSCV control 3T3/v-Src cells but not in Stat3D-expressing 3T3/v-Src fibroblast cells (Fig. 1A, *right*). Multiple bands associated with HIF-1 α in this figure likely result from posttranslational modifications.

We next examined the role of Stat3 in the hypoxic induction of HIF-1 α using A2058 human melanoma cells. Previous

studies established that Stat3 is constitutively activated in these cells due, at least in part, to endogenous c-Src kinase activity (18). To determine the effects of silencing Stat3 on HIF-1 α induction by hypoxia, A2058 cells stably expressing Stat3 short hairpin RNA (shRNA) were created. The Stat3 shRNA-transfected cells had little Stat3 DNA-binding activity as determined by electrophoretic mobility shift assay (Fig. 1B, *right*). When these cells were exposed to either 1% O₂ or CoCl₂, HIF-1 α induction was significantly limited compared with the control cells transfected with a vector encoding scrambled shRNA (Fig. 1B, *left*). To determine whether blocking Stat3 signaling using a pharmacologic Stat3 inhibitor would abrogate hypoxia-induced HIF-1 α up-regulation, A2058 melanoma cells were treated with the Stat3 inhibitor, CPA-7, using conditions previously described (19). As shown in Fig. 1C, blocking Stat3 activity with CPA-7 resulted in pronounced reduction of HIF-1 α protein levels induced by hypoxia. These data show that blocking Stat3 in tumor cells with activated Src kinase can effectively limit HIF-1 α protein induction by hypoxia.

Stat3 Signaling Is Required for HIF-1 α Expression at the RNA Level in Cancer Cells

Our finding that Stat3 affects HIF-1 α protein levels under both normoxia and hypoxia prompted investigation of the possibility that constitutively activated Stat3 is required for HIF-1 α RNA transcription. To determine whether Stat3 might regulate HIF-1 α RNA expression in the activated Src cells, we first did Northern blot analysis of 3T3/v-Src cells transduced with either a control vector (MSCV) or a vector expressing a dominant-negative Stat3 mutant (Stat3D). The 3T3 variants were cultured with either 20% O₂, hypoxia (1% O₂) or hypoxia mimic (CoCl₂). Using probes derived from HIF-1 α cDNA for the Northern blot analysis, HIF-1 α mRNA levels in 3T3/v-Src- and 3T3/v-Src-expressing Stat3D were compared. Although HIF-1 α RNA levels were similar in the 3T3/v-Src cells cultured under different O₂ concentrations due to similar high Stat3 activation, they were greatly inhibited in the cells expressing Stat3D, as determined by both Northern blot analysis. Real-time PCR confirmed that Stat3 contributes to HIF-1 α mRNA up-regulation in 3T3/v-Src cells (Fig. 2A). Similarly, in A2058 human melanoma cells, HIF-1 α mRNA expression is reduced when Stat3 is knocked down by transient transfection of Stat3 small interfering RNA (siRNA), as shown by real-time PCR (Fig. 2B). Further, treating A2058 melanoma cells with a Stat3 inhibitor (CPA-7) using previously described conditions (19) blocked Stat3 activity, resulting in a pronounced decrease in HIF-1 α RNA under both normoxia and hypoxia conditions (Fig. 2C). These findings show that Stat3 is a crucial requirement for the expression of HIF-1 α RNA, suggesting that it is possible to block this transcription factor to effectively inhibit HIF-1 elevation in cancer cells due to both hypoxia and oncogenic signaling. Although Stat3 might be the first key transcription factor identified for this promoter, it is very likely that other transcription factors and cofactors are also critical for HIF-1 α RNA expression. Further understanding of the HIF-1 α transcriptional complex should facilitate molecular targeting to effectively inhibit the HIF-1 oncogenic potential.

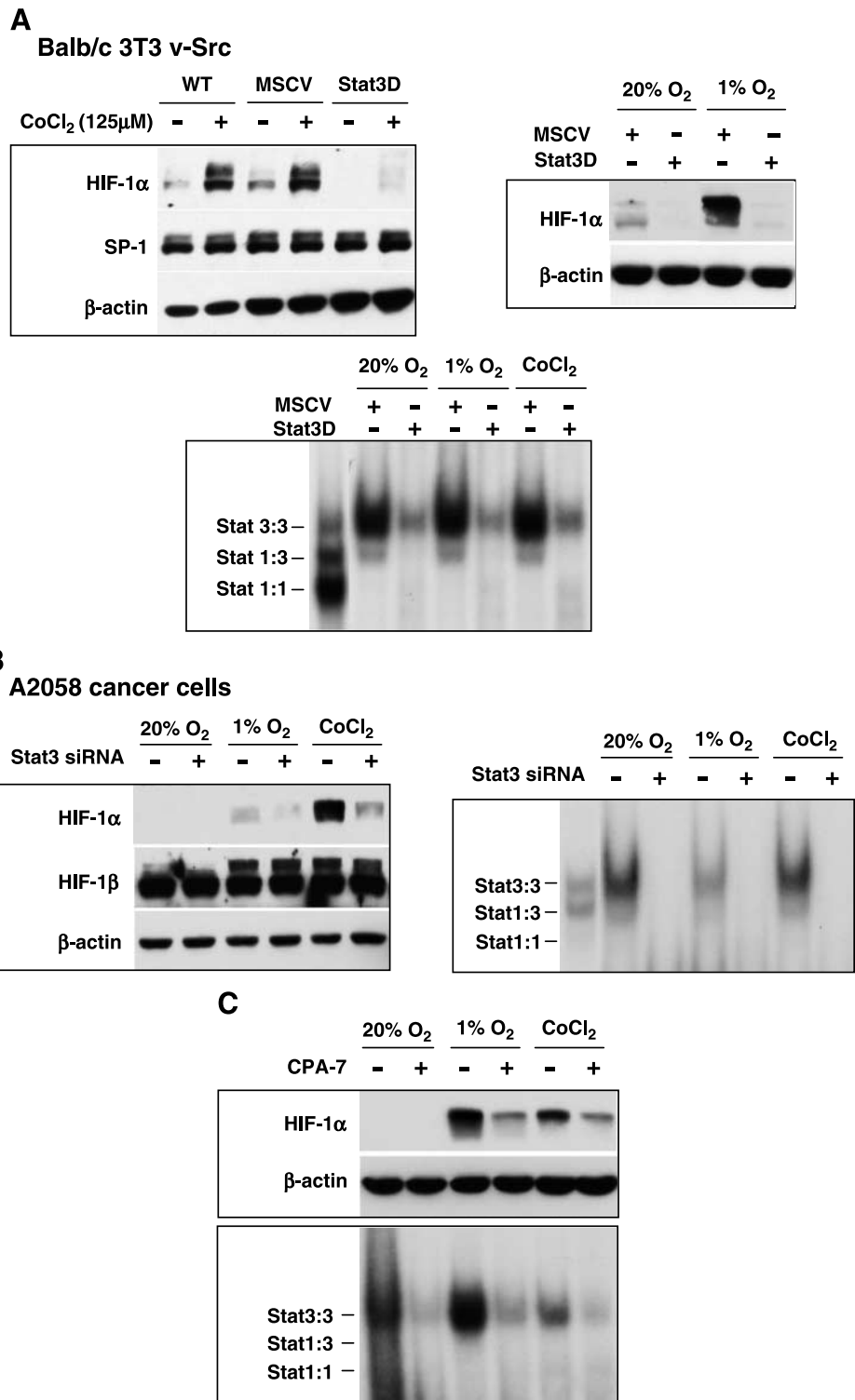


FIGURE 1. Stat3 is required for hypoxia-induced HIF-1 α expression in transformed cells. **A.** 3T3/v-Src, MSCV 3T3/v-Src, and MSCV-Stat3D 3T3/v-Src cells were cultured for 20 h in serum-free medium followed by treatment with hypoxia, using either CoCl₂ (125 μ mol/L) or 1% O₂, for 6 h. Nuclear extracts (30 μ g) were analyzed by Western blotting using the indicated antibodies. SP-1 and/or β -actin were used to verify equal protein loading. Bottom, Stat3 DNA-binding activity in the indicated cells under normoxia (20% O₂) and hypoxia (1% O₂) conditions was determined by EMSA using nuclear extracts. **B.** HIF-1 α induction by hypoxia in human cancer cells is also Stat3 dependent. Human A2058 melanoma cells were stably transfected with empty pSilencer or the same vector encoding Stat3 shRNA, which were described previously (12). Nuclear extracts were analyzed by Western blotting using the indicated antibodies. Right, Stat3 activity determined by EMSA in A2058 cells transfected with either scrambled control siRNA or Stat3 siRNA oligonucleotides, under both normoxia and hypoxia conditions. **C.** A2058 human melanoma cells were treated for 20 h with the Stat3 small-molecule inhibitor CPA-7 (10 μ mol/L) followed by treatment with 1% O₂ or CoCl₂ for 6 h in the presence of this inhibitor. Nuclear extracts were analyzed by Western blot using the indicated antibodies. The inhibitory effects of CPA-7 on Stat3 DNA-binding activity were assessed by EMSA (*bottom*).

HIF-1 α mRNA Level Is Significantly Elevated in Tumor Stromal Myeloid/Immune Cells through Stat3

The importance and mechanisms of HIF-1 α elevation in cancer cells have been intensively investigated. However, whether the tumor microenvironment contributes to HIF-1 α accumulation in tumors remains to be fully explored. We therefore

assessed whether tumor-derived myeloid CD11b⁺ cells (which include macrophages, dendritic cells, and granulocytes among others) have elevated HIF-1 α mRNA compared with their splenic counterparts. Results from real-time PCR indicated that HIF-1 α mRNA is markedly increased in tumor-derived CD11b⁺ cells relative to their normal splenic counterparts (Fig. 3A).

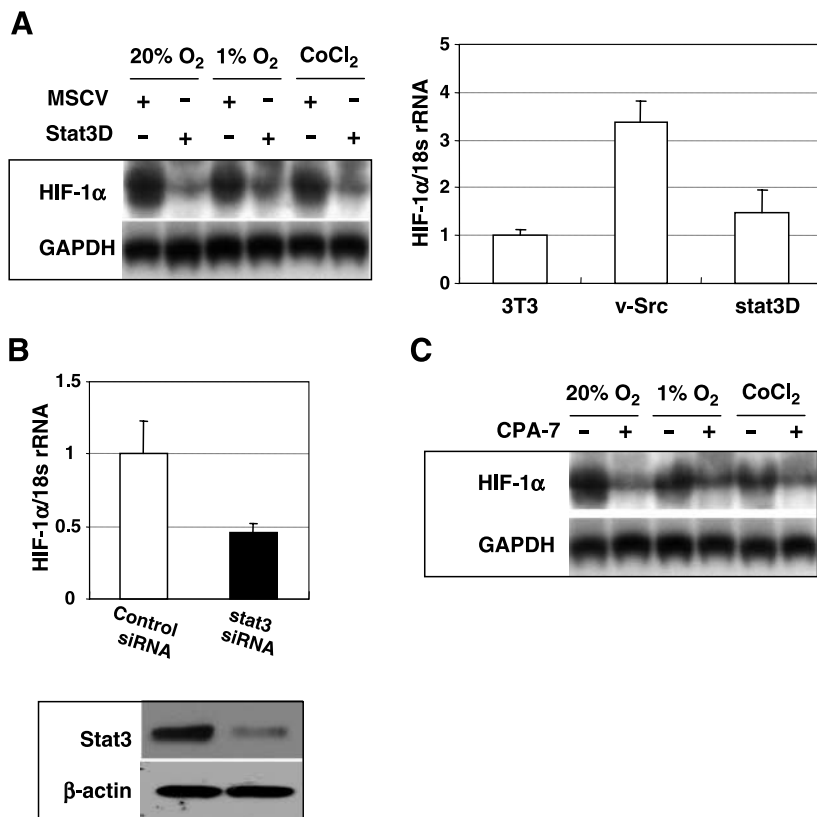


FIGURE 2. Stat3 signaling is required for HIF-1 α expression at the RNA level in transformed cells. **A.** Left, Northern blot analysis of MSCV 3T3/v-Src and MSCV-Stat3D 3T3/v-Src cells. The indicated cells were cultured for 20 h in serum-free medium followed by treatment with 1% O₂ or CoCl₂ (125 μ M/L) for 6 h. Total RNA was collected and HIF-1 α RNA levels were determined by Northern blotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to verify equal loading. Right, confirmation of the Northern blotting results shown on left using real-time PCR ($n = 3$). **B.** HIF-1 α mRNA level is reduced in Stat3 knockdown A2058 melanoma cells. Real-time PCR was done to determine the levels of HIF-1 α mRNA in A2058 tumor cells after transient transfection with either scrambled control siRNA or Stat3 siRNA oligonucleotides. Bottom, the efficiency of Stat3 knockdown as determined by Western blot analysis. **C.** Stat3 inhibitor blocks HIF-1 α RNA expression. Total RNA was prepared from A2058 tumor cells treated as indicated with CPA-7, and HIF-1 α RNA levels were determined by Northern blot analysis.

The generation of mice with *Stat3*^{-/-} immune cells and hematopoietic cells has been described (19). We prepared RNA from CD11b⁺Gr1⁺ (immature myeloid cells), which accumulate in tumors, from B16 melanoma tumors grown in mice with and without *Stat3* alleles in their hematopoietic systems. Real-time PCR analyses showed that HIF-1 α mRNA expression in the tumor stromal immature myeloid cells is dependent on Stat3 activity (Fig. 3B). These results show that components of the tumor microenvironment express high levels HIF-1 α , suggesting an important contributing role of the nontransformed cells to the accumulation of HIF-1 in cancer. These data also show that Stat3 signaling is critical for the expression of HIF-1 α by components of the tumor microenvironment.

Stat3 Directly Contributes to HIF-1 α RNA Expression

To confirm that Stat3 participates in regulation of HIF-1 α at the RNA expression level, we did promoter assays using a HIF-1 α promoter-luciferase vector we constructed. Cotransfecting an expression vector encoding v-Src with the luciferase reporter construct into mouse 3T3 fibroblast cells can increase HIF-1 α promoter activity. Moreover, overexpressing wild-type Stat3 together with v-Src further enhances HIF-1 α promoter activity (Fig. 4A, left). To further characterize the HIF-1 α promoter in terms of Stat3-mediated regulation, we constructed both deletion and site-specific mutants. Site-specific mutations at a Stat3 DNA-binding site (positions -363 to -355) and deletion of 319 bp of 5' of the promoter led to some inhibition of promoter activity (Fig. 4A, right). However, a mutant contains both the 5' end deletion and the Stat3 site-specific mutations lost Stat3-

associated promoter activity (Fig. 4A). These data support the notion that activation of Stat3 signaling in cancer cells, mediated by oncoprotein signaling pathways, contributes to HIF-1 α RNA up-regulation. To show a direct involvement of Stat3 in the HIF-1 α transcription complex, chromatin immunoprecipitation assays were done. In 3T3/v-Src cells, which contain constitutively activated Stat3 (Fig. 1A), Stat3 binds to the HIF-1 α promoter in a region containing consensus Stat3 DNA-binding sites (Fig. 4B). To investigate the possible direct involvement of Stat3 in regulating expression of the *HIF-1 α* gene in tumors *in vivo*, we next performed a chromatin immunoprecipitation assay using extracts of whole tumor tissues. B16 melanoma tumors were harvested and chromatin extracts were prepared followed by chromatin immunoprecipitation assays as previously described (20). Results from these experiments show that Stat3 protein binds to the HIF-1 α promoter in growing tumors (Fig. 4B), indicating a direct involvement of Stat3 protein in regulating HIF-1 α promoter activity.

Discussion

The importance of HIF-1 in tumor progression and resistance to various therapies has been well established. Due to the complexity of its regulation, which operates both at protein stability and protein synthesis levels, targeting HIF-1 for cancer therapy remains a major challenge. Published results show that Stat3 is critical for HIF-1 α protein synthesis induced under growth stimulation via diverse oncogenic signaling pathways. In the current study, we show that in both v-Src-transformed cells and cancer cells with activated c-Src, HIF-1 α

protein level under hypoxia condition is also Stat3 dependent. Importantly, our results reveal a novel mechanism by which HIF-1 α is regulated. The fact that blocking Stat3 can inhibit HIF-1 α RNA transcription provides an opportunity to effectively inhibit HIF-1 oncogenic potentials.

Recently, a critical role of the tumor microenvironment in promoting cancer progression and resistance to therapies has been well recognized. In addition, the importance of inflammation in carcinogenesis is increasingly evident. However, whether tumor stromal cells have increased HIF-1 level remains largely unknown. Our results show that myeloid cells derived from growing tumor have greatly increased HIF-1mRNA level compared with their counterparts from normal spleen. These results suggest Stat3 can up-regulate HIF-1 α in tumor-associated myeloid cells, which in turn can contribute to tumorigenesis. A role of tumor-associated macrophages and myeloid-suppressive cells, both of which are CD11b⁺ cells, in tumor angiogenesis and metastasis is known. Our results suggest that up-regulation of diverse angiogenic and metastatic factors by the tumor-associated myeloid cells may be partially mediated by HIF-1, whose up-regulation is contributed by Stat3 activation.

It is well established that normal growth signaling by cytokines and growth factors leads to Stat3 activation. Because

many of these normal growth signaling pathways, such as c-Src, epidermal growth factor receptor, human epidermal growth factor receptor 2, and interleukin-6 receptor, are overactive in diverse cancers, Stat3 is constitutively activated at very high frequencies (8). Our recent studies have also shown that Stat3 is persistently activated in immune/myeloid cells in the tumor stroma (11, 19). Although it has been shown that HIF-1 α regulation mainly occurs at the protein synthesis and stability levels, it is conceivable that Stat3-regulated HIF-1 α RNA increase in cancer cells and myeloid cells/inflammatory cells in the tumor microenvironment contributes to the overall levels of HIF-1 α protein in cancer. Regardless whether Stat3-associated HIF-1 α RNA is critical for the overall HIF-1 α protein level in cancer, our finding that Stat3 is required for HIF-1 α expression at RNA level suggests that targeting a key transcription factor involved in HIF-1 transcriptional regulation can affect HIF-1 oncogenic activity under both growth and hypoxia conditions.

Materials and Methods

Cell Lines and Retrovirus Infection

BALB/c v-Src 3T3 fibroblasts cells were grown in DMEM supplemented with 5% calf serum. A2058 human melanoma cells were maintained in RPMI supplemented with 10% fetal bovine serum. Two retrovirus-producing cell lines [Stat3D and MSCV (the control retrovirus vector)] were generous gifts from Dr. D. Link (Washington University, Seattle, WA).

Stat3 shRNA and siRNA

Both shRNA-expressing plasmid vector and siRNA oligonucleotides were used in the study. For the plasmid expression vector, the nucleotide sequence for Stat3 shRNA was 5'-GATCCCCTCAGGTTGCTGGTCAAATTTCAAGAGAATTTGACCAGCAACCTGACTTTTTGGAAA-3' and the negative control (scrambled sequence) was 5'-GATCCACTACCGTTGTTATAGGTGTTCAAGAGACACCTATAACAACGGTAGTTTTTGGAAA-3', respectively (oligonucleotides obtained from Dharmacon RNA Technologies). These two oligonucleotides were inserted into pSilencer hygro shRNA expression vectors (Ambion). After transfecting plasmids into A2058 cells, cells were selected with 300 μ g/mL hygromycin. For real-time PCR of human A2058 cells, scrambled control siRNA and human Stat3 siRNA were obtained from Santa Cruz Biotechnology. The siRNA was transfected at a final concentration of 50 nmol/L using Lipofectamine 2000 (Invitrogen). Total RNA was extracted 48 h after transfection.

Preparation of Protein Extracts and Western Blot Analysis, Electrophoretic Mobility Shift Assay

These procedures were done as previously described (7).

Isolation of CD11b⁺ Cells

The experimental procedures involving mice were done under pathogen-free conditions in accordance with established institutional guidance and approved protocols from Institutional Animal Care and Use Committee of Beckman Research Institute at City of Hope National Medical Center. We obtained *Mx1-Cre* mice from The Jackson Laboratory, and *Stat3^{fllox/fllox}*

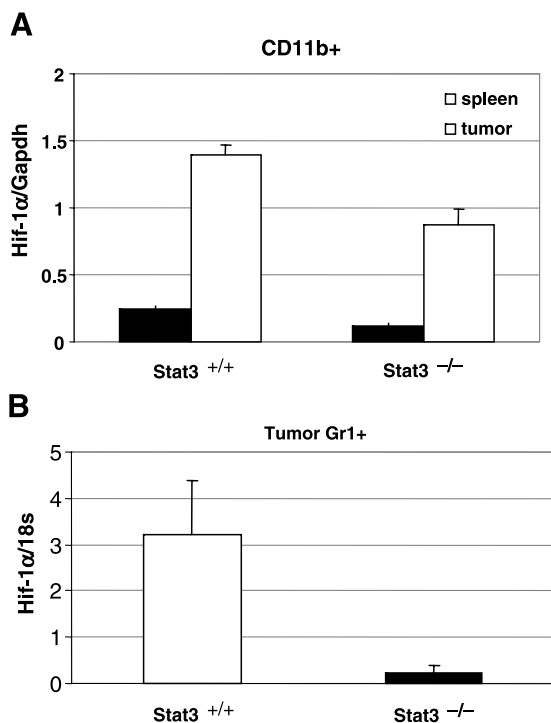


FIGURE 3. Stat3 activity affects HIF-1 α expression in tumor-derived inflammatory cells. **A.** Total RNAs prepared from CD11b⁺ cells isolated from growing B16 tumors and normal spleens from the same mice were subjected to real-time PCR analysis. One group of mice had intact *Stat3* alleles and the other underwent induced ablation of *Stat3* alleles in their hematopoietic system as previously described (19). Real-time PCR was used to detect HIF-1 α mRNA. **B.** Gr1⁺ myeloid cells were isolated from tumors grown in mice with or without *Stat3* in their hematopoietic systems. Either GAPDH or 18S rRNA was used as an internal control for the real-time PCR assays. Columns, mean ($n = 3$); bars, SD.

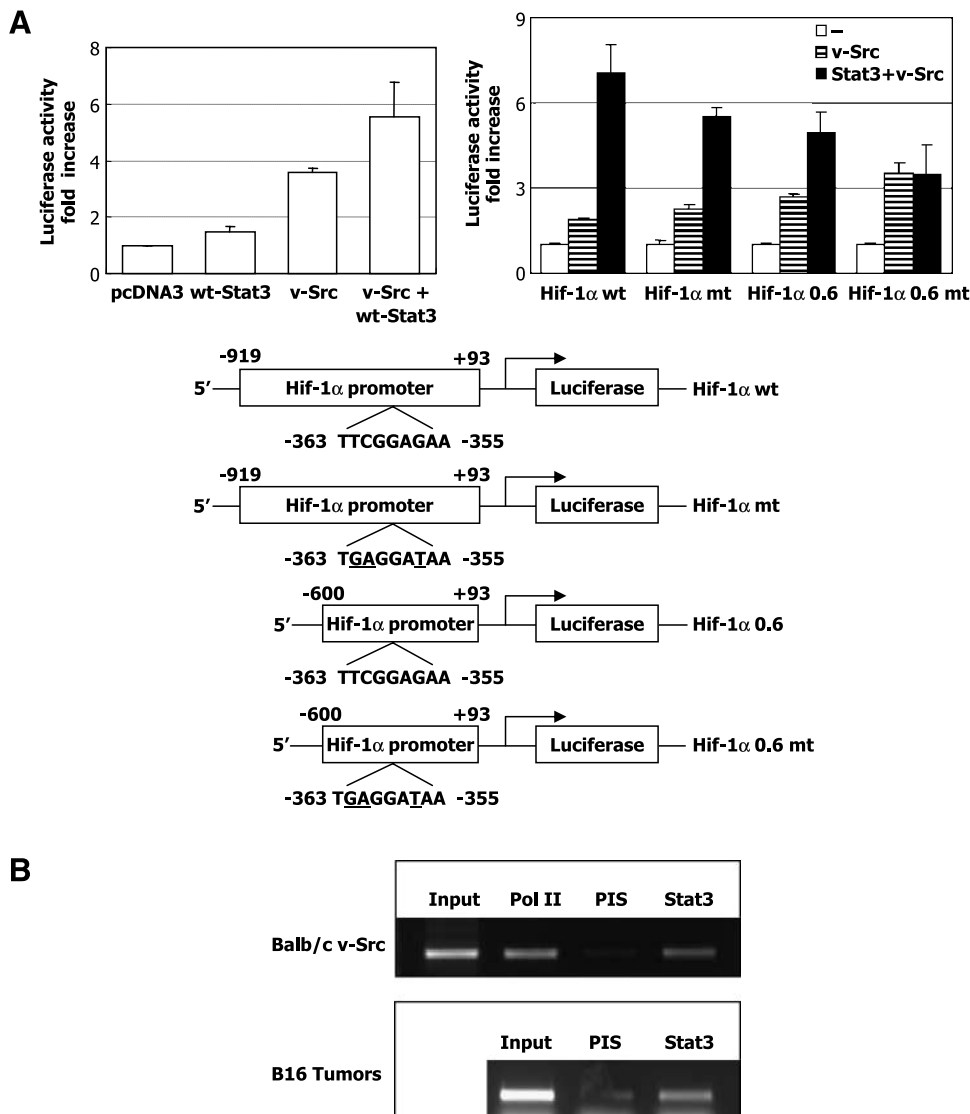


FIGURE 4. Stat3 protein directly binds to the HIF-1 α promoter and contributes to HIF-1 α transcriptional regulation. **A.** Stat3 activity enhances HIF-1 α promoter activity. BALB/c fibroblasts were transiently transfected with empty vector (pcDNA3), a plasmid vector encoding WT-Stat3, a plasmid vector expressing v-Src, or both WT-Stat3 and v-Src expression vectors. A luciferase reporter under control of the HIF-1 α promoter, as well as a *Renilla* luciferase reporter plasmid (pRL-TK internal control), were included in all the transfections. The luciferase activity associated with control pcDNA3 plasmid cotransfection was assigned as 1 (left). Derivatives of the wild-type luciferase HIF-1 α promoter reporter construct, which include those with Stat3 site-specific mutations, deletion mutation, and the deletion mutation with Stat3 site-specific mutations (bottom right) were also analyzed for luciferase activity (top right). **B.** Chromatin immunoprecipitation assays show Stat3 binding to the HIF-1 α promoter in both cultured transformed cells (top) and in growing tumors (bottom). Chromatin extracts prepared from 3T3/v-Src cells and freshly harvested B16 tumors were immunoprecipitated with Stat3 antibody, Pol II antibody, or preimmune serum (PIS). Pol II antibody was used as a positive control and preimmune serum was used as a negative control.

mice were gifts from Drs. Shizuo Akira and Kiyoshi Takeda of Osaka University, Osaka, Japan. Generation of mice with *Stat3*^{-/-} hematopoietic cells by inducible *Mx-Cre* recombinase system has been reported elsewhere (19). Tumor challenges were done in *Stat3*^{fllox/fllox} or *Cre/Stat3*^{fllox/fllox} mice 5 d after poly(I:C) treatment, which induces Stat3 ablation mainly in the hematopoietic system. Two to three weeks after tumor challenge, mice were killed and spleens and tumor specimens were harvested. Purification of splenocyte or specific immune subsets was described previously (19). Protein and RNA were prepared from isolated immune cells, whole spleens, and whole tumors for various analyses as indicated.

RNA Isolation and Northern Blotting

Total RNA was isolated using TRIzol reagent (Invitrogen). Twenty micrograms of RNA were electrophoresed on 1% agarose-formaldehyde gel and transferred to nylon membrane. For preparation of the HIF-1 α probes (both human and mouse), HIF-1 α cDNA was ³²P-radiolabeled using All-in-One random

primer DNA labeling mix (Sigma) according to the manufacturer's protocol.

Real-time Reverse Transcription-PCR

RNA was isolated from cells using the RNeasy kit (Qiagen), and cDNA was produced using the iScript cDNA Synthesis Kit from Bio-Rad. cDNA (100 ng) generated from 1 μ g of total RNA was then used with the iQ SYBR Green Supermix (Bio-Rad). Reactions were quantified using the Chromo4 Real-time Detector (Bio-Rad). The following reverse transcription-PCR primer sets for real-time PCR were purchased from SuperArray: human HIF-1 α (PPH01361A), mouse HIF-1 α (PPM03799A), human 18s rRNA (PPH05666A), mouse 18s rRNA (PPH57735A), and mouse glyceraldehyde-3-phosphate dehydrogenase (PPM02946A). Standard curves of cDNA from samples were used to calibrate the threshold cycle of the relative quantity for each sample. Relative HIF-1 α mRNA level was normalized to the value obtained from control reactions (18S rRNA or glyceraldehyde-3-phosphate dehydrogenase).

Samples were analyzed in triplicate and expressed as the mean (\pm SD).

HIF-1 α Promoter Cloning, Mutagenesis, and Luciferase Assays

Putative HIF-1 α regulatory sequences from 919 bp upstream to 93 bp downstream of the HIF-1 α transcription start site were cloned by genomic PCR using human genomic DNA as a template. The sequences of forward and reverse primers were 5'-GGGACGCGTAGTAGCTTGCAAAGTTGCCAA-3' and 5'-GGGAAGCTTCTCTCCTCAGGTGGCTTGTC-3', respectively. The resulting DNA fragment was cloned into the Topo TA vector. The HIF-1 α promoter fragment was then subcloned into the luciferase reporter vector pGL3 using *Mlu*I and *Hind*III restriction sites that were incorporated into the cloning oligonucleotides. To construct HIF-1 α promoter mutant luciferase reporter plasmids, the Stat3 DNA-binding motif at position -363 to -355 of the wild-type HIF-1 α promoter was mutated using PCR-based site-directed mutagenesis (normal, TTCGGAGAA; mutant, TGAGGATAA). The HIF-1 α 0.6 vector was made by amplifying sequence from -600 to +93 bp of the HIF-1 α promoter and resubcloned into pGL3 plasmid. The PCR primers are 5'-GGGACGCGTACACATCTGAGCAACGAGACC-3' and 5'-GGGAAGCTTCTCCTCAGGTGGCTTGTC-3'. The Stat3 DNA-binding site of HIF-1 α 0.6 vector was also mutated using the same method as described above. All the HIF-1 α promoter plasmids were verified by DNA sequencing.

Transfections were done using the Lipofectamine reagent (Invitrogen) according to the manufacturer's protocol. Briefly, the transfection mixture contained a total of 1 μ g of DNA per 35-mm dish, including 0.2 μ g of firefly luciferase reporter plasmid, 20 ng of *Renilla* luciferase reporter plasmid (pRL-TK internal control), and 0.8 μ g of the test expression vectors or empty control vectors. Cells were harvested 48 h after transfection, and luciferase assays were done using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's protocol. To control for transfection efficiency, firefly luciferase values were normalized to the values for *Renilla* luciferase.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation for cultured cells was done based on the protocol from Upstate Biotechnology. Tissue chromatin immunoprecipitation assays were done as described (20) with some modification. Briefly, nuclei were isolated from B16 tumors and cross-linked to DNA by adding formaldehyde directly to nuclear suspension. The nuclei were pelleted after 1-h incubation at room temperature and then lysed for chromatin preparation. Rabbit anti-Stat3 (C-20, Santa Cruz Biotechnology) and anti-RNA polymerase II (clone CTD4H8, Upstate Biotechnology) antibodies were used for immunoprecipitation. Specific PCR primers were used for a 300-bp region (-565 to -266), spanning the Stat3 binding site (-373) of the HIF-1 α promoter as follows: HIF-1 α forward (+) 5-TCCCTTGTTGTGTAGCAAAGAGCG-3 and HIF-1 α reverse (-) 5'-AATG-

GAAAGCTGCGTCTGGCTTTG-3'. The final products were resolved on 1.5% agarose gels.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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