Molecular and Cellular Pathobiology

Myc Posttranscriptionally Induces HIF1 Protein and Target Gene Expression in Normal and Cancer Cells

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Abstract
c-Myc is frequently overexpressed in tumors and plays an important role in the regulation of cancer metabolism. Hypoxia-inducible factor-1 (HIF1), the master regulator of the hypoxic response, enhances tumorigenesis and influences metabolism via upregulation of the glycolytic pathway and suppression of mitochondrial respiration. Together, deregulated Myc and HIF1 cooperate to lend metabolic advantages to proliferating cancer cells and contribute to the Warburg effect. Here we show that overexpression of Myc significantly stabilizes the α subunit of HIF1 (HIF1α) under normoxic conditions and enhances HIF1α accumulation under hypoxic conditions in cells. Posttranscriptional regulation of HIF1α by Myc led to the induction of HIF1 gene targets. Normoxic HIF1α protein expression was also dependent on Myc. Functionally, HIF1α expression was required for Myc-induced anchorage-independent growth and cell proliferation. Myc-dependent stabilization of HIF1α involved either disruption of binding to the VHL complex or posttranslational protein modifications. Taken together, our findings uncover a previously uncharacterized regulatory relationship between Myc and HIF1 that has important implications for cancer metabolism and development. Cancer Res; 72(4); 1–9. ©2011 AACR.

Introduction
Breast cancer is the most common malignancy of women worldwide and targets 13% of women in the United States (National Cancer Institute). The c-myc oncogene, which encodes the transcription factor c-Myc, contributes to many forms of cancer and was one of the first genes found to be amplified in breast cancer (1). Studies suggest that at least 15% to 20% of breast cancers present with significant amplification of c-myc and that overexpression of c-Myc likely occurs in an even greater number of tumors through other mechanisms (2). Animal model studies have confirmed that constitutive Myc expression in the mammary gland is oncogenic in transgenic mice (3). Microarray studies have exposed Myc as a weak yet pleiotropic transcription factor, possessing the ability to activate or repress 5% to 10% of all genes by 1.5-fold or more (4). Whereas these target genes vary greatly depending on the cellular context, commonly targeted processes include the cell cycle, metabolism, and apoptosis (5).

In transformed cells, Myc promotes glycolysis by activating glycolytic enzymes (6, 7). Tumor cells often exhibit increased rates of glycolysis, even in the presence of adequate oxygen concentrations (the Warburg effect); although, increased rates of glycolysis normally occur under hypoxic conditions (8, 9). Hypoxia-inducible factor-1 (HIF1) is the key mediator of the hypoxic response in cellular tissues, comprised of a constitutively expressed HIF1β subunit and an oxygen-responsive HIF1α subunit (10, 11). In oxygenated tissues, HIF1α has a very short half-life (10, 12). Rapid turnover is mediated through the hydroxylation of two prolines at amino acids 402 and 564, performed by oxygen-dependent prolyl hydroxylases (13–15; reviewed in ref. 16). Prolyl-hydroxylation of HIF1α leads to ubiquitination by the Von Hippel–Lindau (VHL) E3 ubiquitin ligase and degradation via the 26S proteasome (reviewed in refs. 17, 18). In a hypoxic environment, HIF1α becomes stabilized (19, 20), although, in some instances, HIF1α can be stabilized under normoxia as well (reviewed in refs. 21, 22). HIF1α is commonly expressed in cancers, as tumor microenvironments suffer from hypoxia owing to an incomplete vasculature.

Studies have reported HIF1-induced opposition of Myc under hypoxia when Myc is expressed at nontumorigenic levels. Studies have also revealed the cooperation of HIF1 and deregulated Myc under hypoxia (23–26). However, our data show that Myc regulates HIF1α expression under both normoxia and hypoxia, and that HIF1α expression represents an important component of Myc function. Our results show a novel and complex relationship between Myc, HIF1α, and tumor development.
Materials and Methods

Cell culture, transfection, and retroviral infection

Retroviral producing PhoeNX cells and breast cancer lines MCF7 and T47D were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS. IMEC epithelial cells were cultured in 1:1 DMEM/F12 medium supplemented with 5% FBS and other factors (27). Retroviral infection was carried out using PhoeNX cells. Briefly, PhoeNX cells were transfected with 7 μg DNA using Lipofectamine 2000 (Signagen) according to manufacturer’s instructions. Virus was harvested from the culture medium 2 days later and used to infect recipient IMECs. Cells were selected 2 days later and maintained in 150 μg/mL hygromycin (Calbiochem) to select for LXSH vectors (LXSH, LXSH-Myc). Hypoxic conditions were induced by culturing cells for 2 hours in a sealed hypoxia chamber (Billups Rothenberg) after flushing with a mixture of 1% O2, 5% CO2, and 94% N2. Hypoxic conditions were also induced by the addition of CoCl2 (Sigma) at a concentration of 200 μM for 3 hours. We refer to the ambient oxygen concentration as normoxic (95% air, 5% CO2). Although the ambient oxygen concentration is not the physiologic normoxic state, this is a frequently used convention in the literature.

Cell counts

Cells were plated at 1 x 10^4 per well in 6-well plates on day 0. Cells were maintained as stated earlier. Cells were harvested each day for 4 days and counted using a hemacytometer. At least 100 cells were counted. The experiment was conducted in duplicate on 2 separate occasions. Knockdown cell growth curves were achieved by plating cells at 1 x 10^4 per well, transfecting with HIF1α and nontargeting control siRNA 24 hours later (day 1), and counting cells for 3 days posttransfection. The experiment was conducted in duplicate on 2 separate occasions.

Soft agar assay

Assays were done in 6-well plates in duplicate on 2 separate occasions. The lower layer of agar consisted of 2 mL IMEC medium containing 10% FBS and 0.6% Noble Agar (USB). The upper layer consisted of 2 mL IMEC medium containing 10% FBS, 0.3% Noble Agar, and 4 x 10^5 cells. Agar at 50°C was mixed with medium at 37°C, plated, and left to set for 10 minutes. Plates were fed every other day with 250 μL IMEC medium containing 10% FBS. After 10 days, undivided cells and colonies were scored using a reticle, measuring the greatest diameter. A total of 100 cells were counted per well.

Real-time PCR

RNA was extracted from log-phase cells using TRIzol (Invitrogen) according to manufacturer’s instructions. RNA was converted into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad) and PCR was done using iQ SYBR Green Supermix and the CFX96 Real-Time PCR Detection System (Bio-Rad). Primers used were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) GCTCAGACACCATGGGAAGGTGAG and GCTGATGATCAGGCTTGTTGFCATAC; Myc CGAGGAGGA-GAATCTACCAGC and CGAGAAGCCTGCACCATACAG-TCC; HIF1α ACATGTATCCAGCAATCTCAAA and CCTACTGT-TGAAAGAAGTGGA; HK2 GGATGGTAGCTGCTGATCTGC and GGAACCTCAGGCTTCCTGCTGCC; ALDOA CCATGCCCCTACCAA-TATCCAGC and GGTCGAGATCCATCTCCCTGGCC; eNOS GGAGAGATCCACCTCAGTGAAC and CTTTGTCTCTCAAT-GTATGC; BGN GGAACATGAACTGATCGAGATGG and CGATCATCTGATCTGGTTGTCG; VHL CCTTGTGCTCTT-CAGAGATGC and GTGCTTGTCACATCTGGTTGTCG; CUL2 CGACAGAATCAACACTACACTTGCC and CCTTGCTGTAT-TCTTCGAGGACTCC; RBX1 GACAGCATGAGGATGTTGATGATACC and CCTTGCTGTGTTTGGACCCAGC; Elongin B CCACAGAC-CACCATCTCCAGC and CGTGCTGTGTTGACTGGTGAAGC; Elongin C CCTATGGTGCGTGTAGGACC and GCAGTT-CCAGTCAATTCAGG.

Western blots

Cells were lysed in F-Buffer and cell extracts were normalized for protein content. Proteins were resolved by SDS-PAGE, transferred to polyvinylidine difluoride membrane, and immunoblotted with antibody in 5% milk. Antibodies used include monoclonal anti-HIF1α (Novus Biologicals), polyclonal anti-HIF1α (Novus Biologicals), monoclonal anti-VHL (BD Biosciences), polyclonal anti-VHL (Novus Biologicals), anti-hydroxyproline-564–specific-HIF1α (kindly provided by R. Freeman), monoclonal anti–c-Myc antibody (C-33; Santa Cruz), polyclonal anti–c-Myc antibody (N262; Santa Cruz), anti–ß-tubulin (Santa Cruz), monoclonal anti-CUL2 (Santa Cruz), polyclonal anti-CUL2 (Abcam), polyclonal anti-Rbx1 (Santa Cruz), polyclonal anti-Elongin B (Santa Cruz), monoclonal anti-Elongin C (BD Biosciences), and polyclonal anti-Elongin C (Novus Biologicals). Immunoprecipitations were carried out by complexing Protein A/G-Plus Agarose beads with 1 μg of antibody for 1 hour and then mixing the beads with cell extract overnight. ImageJ (NCBI) was used to quantitate protein expression levels.

Cycloheximide assay

Cells were plated in 6-well plates and treated with cycloheximide (CHX) 24 hours postplating. CHX was added at a concentration of 50 μg/mL to the cells at 20-minute intervals. Hypoxic extracts were made by treating cells with 200 μM/L CoCl2 for 3 hours prior to CHX treatment. Protein extracts were prepared as previously described. HIF1α and Myc half-lives were calculated after quantitating protein and normalizing to β-tubulin.

siRNA

Experiments involving siRNA were conducted by transfecting HIF1α, c-myc, or nontargeting siRNA (Dharmacon) at 2 μM/L in 6-well plates according to manufacturer’s instructions. Cells were replated for 24 hours following transfection for soft agar analysis. RNA and protein was harvested for analysis 24 or 48 hours following transfection. siRNA growth curve experiments were conducted by transfecting HIF1α and nontargeting siRNA at 2 μM/L in 24-well plates according to manufacturer’s instructions.
Results

Myc enhances accumulation of hypoxia regulator HIF1α

We have previously shown that overexpression of Myc in TERT-IMEs leads to a partial oncogenic transformation with an increased proliferation rate and anchorage-independent growth (28). It is important to note that Myc expression in the transduced IMEs is similar to Myc protein levels found in breast cancer cell lines T47D and MDA-231 and, hence, represents physiologically relevant expression (28). Whole genome expression analysis revealed that the most significantly enriched Myc-induced cluster corresponded to angiogenesis and vascularization (Supplementary Table; data available on request). Previous studies have linked angiogenesis to Myc in a transgenic model (29, 30). Furthermore, many genes involved in these pathways have been scored as potential direct Myc target genes in microarrays. However, the most prominent regulator of angiogenesis is HIF1α, which coordinateably controls the expression of a large number of genes in the pathway. Therefore, we explored whether the strong induction of an angiogenic program is due to activation of HIF1α, instead of a direct effect of Myc.

To assess a potential role for HIF1α in the induction of an angiogenic gene response, we examined the levels of HIF1α protein in vector and Myc-overexpressing cells under normoxic (95% air, 5% CO2) or hypoxic conditions (1% O2, 5% CO2, 94% N2). As expected, there were very low levels of HIF1α in IMEcs cultured under normoxic conditions, and the level was induced substantially under hypoxia. Surprisingly, we found that overexpression of Myc enhances the accumulation of HIF1α under normoxic conditions to a significant level. Moreover, overexpression of Myc exaggerates HIF1α accumulation under hypoxic conditions (Fig. 1A). On average, HIF1α expression in Myc-overexpressing cells under normoxia is 10 times more than in vector IMEcs (Fig. 1B). We determined whether the increase in protein expression is due to an increase in transcription of the HIF1α gene by carrying out quantitative real-time (RT) PCR under normoxic and hypoxic conditions. We found that HIF1α is not transcriptionally induced by hypoxia, as expected, or by Myc (Fig. 1C). Myc transcription is also not affected by hypoxia. These data suggested that Myc-enhanced HIF1α expression is posttranscriptionally regulated.

We treated the vector control IMEs and IME-Myc cells with the proteasome inhibitor MG132 to block protein degradation and assess the total amount of HIF1α protein being translated under normoxia and hypoxia. As expected, the enhanced level of HIF1α under hypoxia is an effect of stabilization and not increased translation, as protein levels become equal when degradation is inhibited (Fig. 1D). We found that, even though normoxic HIF1α is stabilized significantly by Myc, treatment with MG132 leads to further stabilization, indicating that there is some ongoing degradation in IMEC-Myc cells. In contrast, all of the HIF1α protein seemed to be fully stabilized under hypoxia in Myc-overexpressing cells because MG132 treatment gives no further enhancement.

Because HIF1α protein levels are primarily controlled by proline hydroxylation and interaction with the VHL E3 ligase, we tested whether the enhanced HIF1α expression in response to Myc under normoxia is due to the loss of proline hydroxylation. We used an antibody specific to the prolyl hydroxylation site (hydroxy-P564 HIF1α) to measure the extent of modification relative to HIF1α protein levels. We confirmed that HIF1α is prolyl hydroxylated in normoxic cells, and hydroxylation is suppressed under hypoxia (Fig. 1A). To compare HIF1α modification with or without high levels of Myc, we treated cells with MG132 to prevent turnover of HIF1α and quantitated the level of prolyl hydroxylation to total HIF1α levels. We find that the ratio of prolyl hydroxylated to total HIF1α is identical with high and low Myc (Fig. 1D and 1E), thus the elevated levels of HIF1α are not due to the suppression of proline hydroxylation. Furthermore, prolyl hydroxylation is sensitive to reactive oxygen species (ROS), but quenching of ROS with N-acetylcysteine had no effect on the Myc-dependent accumulation of HIF1α (Supplementary Figure).

We wanted to explore whether HIF1α protein expression is induced by Myc in other cell types. We measured protein levels in myc−/− Rat1 fibroblasts before and after stable reconstitution with mouse c-Myc (Fig. 1F). As in IMEs, HIF1α protein levels are enhanced with Myc reconstitution under normoxic conditions, and Myc dramatically exaggerates HIF1α levels in hypoxia. As expected, there are no changes in HIF1α mRNA levels due to Myc expression (Fig. 1G).

HIF1α induces its target gene expression

We next looked at the expression levels of HIF1α target genes to determine whether there is a functional downstream effect of enhanced HIF1α by Myc under normoxia in IMEcs. We transiently transfected vector and Myc-overexpressing IMEcs with siRNAs against HIF1A, c-myc, or a nontargeting control and achieved depletion at the RNA level (Fig. 2A). By RT-PCR, we found that glycolytic enzymes HK2 and ALDOA, nitric oxide synthase eNOS, and collagen fibril assembly proteoglycan BGN are all transcriptionally upregulated in Myc-overexpressing cells under normoxia (Fig. 2B). Of these genes, HK2 is the only one known to be a direct binding target of both HIF1α and Myc. Consistent with this, these genes are downregulated in response to HIF1α depletion. These genes are also all downregulated in response to Myc knockdown, suggesting that there is indeed a functional downstream effect of enhanced HIF1α expression by Myc.

Myc induces HIF1α expression in breast cancer cells

We also explored whether HIF1α protein levels are dependent on Myc in human breast cancer cells. To this end, we examined HIF1α and Myc in the breast cancer cell lines T47D and MCF7 (Fig. 3A). MCF7 cells have 3.5-fold higher Myc protein levels than T47D and have significant constitutive HIF1α protein under normoxic conditions, whereas T47D cells have virtually undetectable levels (Fig. 3B). As in other cell types, Myc protein

ON-TARGETplus Human HIF1A siRNA: J-004018-07-0010, J-004018-08-0010
ON-TARGETplus Human c-myc siRNA: J-003282-23-0005, J-003282-24-0005
ON-TARGETplus Nontargeting siRNA: D-001810-01-05
levels largely correspond to mRNA levels, whereas HIF1α mRNA levels are equivalent between cell lines (Fig. 3C). To test whether HIF1α protein levels are Myc dependent in MCF7 cells, we used siRNA to deplete native Myc. We found that the loss of Myc led to a corresponding reduction in HIF1α, suggesting that HIF1α expression is dependent on Myc in MCF7 cells (Fig. 3D).

**Myc stabilizes HIF1α protein**

Because we found elevated HIF1α protein with no change in mRNA levels (Fig. 1), we hypothesized that the substantial accumulation of HIF1α in response to Myc under normoxia and hypoxia could be due to a decrease in its rate of degradation. We treated IMECs and IMEC-Myc cells with CHX to block protein synthesis and assessed HIF1α protein levels at 20 minutes intervals under both normoxic and hypoxic conditions (Fig. 4). As previously described, HIF1α has an extremely short half-life under normoxia (t1/2 < 7 minutes) and is barely detectable in the vector cells. However, we found that overexpression of Myc under normoxic conditions significantly stabilizes HIF1α protein (t1/2 = 56 minutes). This stabilization can account for most or all of the enhanced HIF1α protein levels in IMEC-Myc. In contrast, Myc protein turnover is similar between control and Myc-overexpressing cells. Under hypoxia, HIF1α protein is stabilized (t1/2 = 42 minutes) as previously described (11, 29, 30). However, Myc-overexpression further stabilizes HIF1α (t1/2 > 90 minutes), which can account for the hyperinduction of HIF1α in hypoxic IMEC-Myc cells.

**Myc enhances accumulation of VHL complex components**

We next sought to determine other possible mechanisms that could account for Myc-induced stabilization of HIF1α protein. Because HIF1α prolyl hydroxylation is maintained in IMEC-Myc cells (Fig. 1E), we turned our focus to the next step in the normoxic regulation of HIF1α: the recognition and ubiquitination of prolyl-hydroxylated HIF1α by the VHL complex. We hypothesized a role for Myc in rendering the VHL complex less functional. We considered that Myc could be in the normoxic regulation of HIF1α protein expression and then normalized to GAPDH. F. Western blot analyses of myc−/− rat fibroblasts (vector or Myc reconstituted) were done as described above. G. real-time RT-PCR was carried out using RNA harvested from myc−/− rat fibroblasts and primers specific to HIF1α, c-myc, and GAPDH in triplicate. Average c-myc and HIF1α RNA levels are normalized to GAPDH. HIF1α is normalized to its normoxic vector. c-myc is normalized to normoxic myc−/− (Myc) as vector cells express no c-myc RNA. Error bars, SD.

**Figure 1.** Myc posttranscriptionally enhances accumulation of HIF1α under both normoxia and hypoxia. A, Western blot analyses of IMECs were done with equivalent amounts of protein using anti-HIF1α antibody, anti-hydroxyl P564-specific HIF1α antibody (OH-P564), anti-c-Myc antibody, and anti-β-tubulin antibody. Hypoxic cell extracts were made after incubating cells for 2 hours in a hypoxia chamber with 1% oxygen. Normoxic HIF1α (OH) blots are shown at a longer exposure than the hypoxic HIF1α blots. Norm, normoxia, Hypo, hypoxia. B, average HIF1α protein expression, normalized to β-tubulin, was quantitated using 3 independent cell extract sets. Expression levels are normalized to normoxic vector. C, real-time RT-PCR was done using RNA harvested from IMECs and primers specific to HIF1α, c-myc, and GAPDH in triplicate. Average c-myc and HIF1α RNA levels are normalized to GAPDH and then normalized to their respective normoxic vectors. D, IMECs were treated with proteasome inhibitor MG132 under normoxic and hypoxic conditions. Western blot analyses were carried out with equivalent amounts of protein using anti-HIF1α and anti-β-tubulin antibodies. E, after treatment with MG132, average hydroxy-P564 HIF1α protein expression was determined in comparison with total HIF1α protein expression under normoxia and hypoxia. F, Western blot analyses of myc−/− rat fibroblasts (vector or Myc reconstituted) were done as described above. G. real-time RT-PCR was carried out using RNA harvested from myc−/− rat fibroblasts and primers specific to HIF1α, c-myc, and GAPDH in triplicate. Average c-myc and HIF1α RNA levels are normalized to GAPDH. HIF1α is normalized to its normoxic vector. c-myc is normalized to normoxic myc−/− (Myc) as vector cells express no c-myc RNA. Error bars, SD.
As with HIF1\(\alpha\) regulation seemed to be entirely posttranscriptional (Fig. 5B). Furthermore, except for a 2-fold induction of components under both normoxic and hypoxic conditions (Fig. 5A). Enhances the accumulation of all of the VHL complex components, which would destabilize complex formation under both normoxic and hypoxic conditions. As in IMECs, the protein levels of the VHL components are enhanced in response to elevated Myc expression under both normoxic and hypoxic conditions (Fig. 5C). Of note, both VHL and Elongin B not only change in abundance but also shift in migration in SDS-PAGE (Fig. 5A and C), suggesting that there may be Myc-induced changes in protein modification.

**HIF1\(\alpha\) exhibits reduced binding to the VHL complex in Myc-overexpressing cells**

Because we found that HIF1\(\alpha\) is stabilized even in the presence of upregulated VHL complex components, we hypothesized that the VHL complex is less effective at recognizing and targeting HIF1\(\alpha\) for degradation in Myc-overexpressing cells. To assess the association between the VHL complex and HIF1\(\alpha\), we carried out immunoprecipitations with different components (VHL, CUL2, and Elongin C) and with HIF1\(\alpha\). Indeed, we found that there is less binding of HIF1\(\alpha\) to VHL complexes in IMEC-Myc cells compared with vector cells under both normoxia and hypoxia (Fig. 6A). Normoxic association between HIF1\(\alpha\) and CUL2, Elongin C, and VHL are each reduced to only about 30% of the association seen in vector cells. This reduction may account for a significant portion of the HIF1\(\alpha\) stabilization in IMEC-Myc cells. Furthermore, by carrying out immunoprecipitations between

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CUL2, Elongin C, and VHL, we found that there were also apparent changes in VHL complex component associations in IMEC-Myc cells compared with vector cells (Fig. 6B). This suggested that, in addition to associating less with HIF1α, there may be fewer functional VHL E3 ligase complexes formed in Myc-overexpressing cells.

**HIF1α expression is critical for Myc-induced soft agar growth and proliferation**

One of the most important questions we wanted to explore is whether or not HIF1α accumulation contributes to Myc-induced oncogenic transformation. To address this question, we carried out siRNA depletion experiments and assessed their effects on Myc-induced anchorage-dependent growth, which is an established hallmark of transformation. We transiently transfected IMECs and IMEC-Myc with siRNAs against HIF1A, c-Myc, or a nontargeting control. Myc depletion served as a positive control because soft agar growth is Myc dependent, and loss of Myc expression should ablate the ability of the cells to undergo anchorage-independent growth. We achieved depletion at the protein level for both Myc and HIF1α (Fig. 7A and B). Consistent with the Myc dependence of HIF1α accumulation, depleting Myc from the IMEC-Myc cells reduced the HIF1α protein level nearly back to that found in the parental IMECs. We plated transfected cells in soft agar 24 hours posttransfection with siRNA and scored for growth 10 days later. The IMEC-Myc cells transfected with the nontargeting control grew into colonies as expected (Fig. 7C). Depletion of Myc predictably led to an almost complete loss of colony growth. Strikingly, the loss of HIF1α expression was equally as effective in blocking anchorage-independent growth, as HIF1α depletion almost completely eliminated soft agar colony formation (Fig. 7C). Vector control IMECs do not grow in soft agar and are therefore not represented (28). These data showed that expression of HIF1α is a critical component of Myc-induced anchorage-independent growth. In support of these data, multiple studies have shown that the knockdown of HIF1α in tumor cells suppresses tumor growth (33–35).

Beyond anchorage-independent growth, we showed previously that Myc overexpression also enhances the overall proliferation rate of IMECs compared with vector control cells (28). Because of the importance of HIF1α expression for Myc-induced soft agar growth, we considered that HIF1α might also influence proliferation rates. We measured IMEC growth rates after transfection with HIF1A siRNA and compared them with IMECs transfected with nontargeting siRNA under normoxia (Fig. 7D). Depletion of HIF1α expression greatly reduces the

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**Figure 4.** Expression of Myc stabilizes HIF1α protein. IMECs (vector or Myc-overexpressing) were treated with CHX for 20, 40, and 60 minutes. Hypoxic extracts were made by treating cells with 200 μmol/L CoCl2 for 3 hours prior to CHX treatment. Western blot analyses were done with equivalent amounts of protein using anti-HIF1α, anti-c-Myc, and anti-β-tubulin antibodies. Half-lives of HIF1α and Myc were determined after normalization to β-tubulin. Normoxic HIF1α blots are shown at a longer exposure than the hypoxic HIF1α blots.

**Figure 5.** Myc enhances posttranscriptional accumulation of VHL complex components under both normoxia and hypoxia. A, Western blot analyses of IMECs were carried out with equivalent amounts of protein using anti-VHL, anti-CUL2, anti-RBX1, anti-Elongin B, anti-Elongin C, and anti-β-tubulin antibodies. B, real-time RT-PCR was done using RNA harvested from IMECs and primers specific to VHL, CUL2, RBX1, Elongin B (TCEB2), Elongin C (TCEB1), and GAPDH in triplicate. Average RNA levels are normalized to GAPDH and then normalized to their respective normoxic vectors. C, Western blot analyses of myc−/− rat fibroblasts (−/− reconstituted mouse c-Myc) were carried out as described above. Error bars, SD.
The proliferation rate of Myc-overexpressing IMECs, down to the rate of vector control IMECs. HIF1A siRNA also had a smaller inhibitory effect on control IMECs. Thus, the enhanced growth rate of IMEC-Myc cells is highly dependent on elevated HIF1α levels even under normoxia, and the parental cells are also dependent on low basal HIF1α expression. Although our IMECs are nontransformed, these results are consistent with other studies that found that HIF1α knockdown significantly reduces the growth rates of glioma cells and prostate cancer cells, respectively (36, 37).

**Figure 6.** Reduced association of HIF1α with the VHL complex in Myc-overexpressing cells. A, immunoprecipitation and Western blot analyses were done with equivalent amounts of protein from normoxic and hypoxic IMEC extracts. Immunoprecipitations were quantitated using at least 2 independent cell extract sets. The HIF1α immunoprecipitation:WB CUL2 was normalized to total CUL2 protein, and all other immunoprecipitations were normalized to total HIF1α protein. Expression levels were normalized to normoxic vector. B, quantitated immunoprecipitations between VHL complex components were done as described above. Immunoprecipitations were normalized to total CUL2 protein. Error bars, SD. IP, immunoprecipitation.

**Figure 7.** HIF1α expression is affected by Myc levels and is necessary for anchorage-independent and log-phase growth. A and B, IMECs were transfected with nontargeting siRNA or siRNAs (1 or 2) targeting HIF1A or c-myc. Cell extracts were made 48 hours posttransfection. Western blot analyses were done with equivalent amounts of cell extracts using anti-HIF1α, anti-c-Myc, and anti-β-tubulin antibodies. Average Myc and HIF1α protein expression, normalized to β-tubulin, were quantitated using 3 independent cell extract sets. Expression levels for Myc and HIF1α protein were normalized to their respective vectors. C, IMECs transfected with nontargeting siRNA or siRNAs (1 or 2) targeting HIF1A or c-myc were plated in soft agar 24 hours posttransfection. The number of colonies per 100 plated Myc-overexpressing cells were counted and measured after 10 days in 2 independent experiments. Vector cells do not grow in soft agar and are therefore not included in the analysis. D, cell proliferation rates were measured by counting equivalently plated cells that had been transfected at day 1 with either HIF1A or nontargeting siRNA under normoxia. The graph shows the mean cell number for each day for duplicate experiments. Error bars, SD. Similar results were found for independent siRNAs.
Discussion

In this study, we have explored a previously uncharacterized relationship between Myc and HIF1α expression under both normoxic and hypoxic conditions: Myc-dependent induction of HIF1 protein levels. Previous studies have shown interactions between Myc and HIF1 regulation and function, both synergetic and antagonistic. For example, in cells expressing physiologically normal levels of Myc, HIF1 has been found to functionally oppose Myc activity by disrupting Myc repression of CDK1 p21 (38, 39). In addition to antagonizing Myc function through interaction, HIF1 also counters Myc-mediated regulation of mitochondrial biogenesis (4, 25, 40). Whereas Myc itself promotes mitochondrial biogenesis and oxidative phosphorylation, HIF1 activates target genes that impair mitochondrial respiration. On the other hand, HIF1 and deregulated Myc cooperate to induce glycolysis in both normoxic and hypoxic tissues (23, 24, 26, 43). Studies suggest that Myc overexpression disrupts the sensitive stoichiometry normally governing HIF1 regulation of Myc. Normoxic cooperation of these transcription factors contributes to the Warburg effect, a metabolic phenotype of aerobic glycolysis often seen in cancers.

We report that overexpression of Myc induces a dramatic enhancement in the stability of HIF1α protein under normoxia and also increases stability under hypoxia. A previous study reported that knockdown of Myc in a multiple myeloma cell line decreases HIF1α expression (44), but we provide the first evidence that overexpression of Myc in nontransformed epithelial and fibroblastic cells single handedly induces the accumulation of HIF1α. Furthermore, we show that this induction is entirely posttranscriptional and that it results in the upregulation of HIF1α target genes. We show that Myc is also required for the posttranscriptional accumulation of HIF1α protein in MCF7 breast cancer cells. At the functional level, we show that HIF1α expression is essential for both Myc-induced anchorage-independent growth and Myc-induced proliferation.

The mechanism of Myc-induced HIF1α stabilization remains unresolved. On the basis of measurement of HIF1α hydroxy-P564 levels after blocking protein turnover, it seems that there is no reduction in hydroxylation with high Myc expression (Fig. 1E). The primary effect we observed is that Myc posttranscriptionally upregulates the expression of VHL complex components. Furthermore, we find that HIF1α association with the VHL complex in cells overexpressing Myc is substantially reduced compared with vector control cells. The decreased interaction between HIF1α and the VHL complex likely contributes significantly to the enhanced accumulation of HIF1α protein. This could be due to an unknown modification of HIF1α or to inactivation of the VHL complex E3 ligase activity. We find evidence of disruption of VHL complex protein stoichiometry and possible posttranslational modifications of VHL and Elongin B. Further characterization of changes in the VHL complex in response to high Myc expression will be required to resolve these questions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Myc Induces HIF1

Myc Posttranscriptionally Induces HIF1 Protein and Target Gene Expression in Normal and Cancer Cells

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