Resistance to arginine deiminase treatment in melanoma cells is associated with induced argininosuccinate synthetase expression involving c-Myc/HIF-1α/Sp4

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Abstract
Arginine deiminase (ADI)–based arginine depletion is a novel strategy under clinical trials for the treatment of malignant melanoma with promising results. The sensitivity of melanoma to ADI treatment is based on its auxotrophy for arginine due to a lack of argininosuccinate synthetase (AS) expression, the rate-limiting enzyme for the de novo biosynthesis of arginine. We show here that AS expression can be transcriptionally induced by ADI in melanoma cell lines A2058 and SK-MEL-2 but not in A375 cells, and this inducibility was correlated with resistance to ADI treatment. The proximal region of the AS promoter contains an E-box that is recognized by c-Myc and HIF-1α and a GC-box by Sp4. Through ChIP assays, we showed that under noninduced conditions, the E-box was bound by HIF-1α in all the three melanoma cell lines. Under arginine depletion conditions, HIF-1α was replaced by c-Myc in A2058 and SK-MEL-2 cells but not in A375 cells. Sp4 was constitutively bound to the GC-box regardless of arginine availability in all three cell lines. Overexpressing c-Myc by transfection upregulated AS expression in A2058 and SK-MEL-2 cells, whereas cotransfection with HIF-1α suppressed c-Myc–induced AS expression. These results suggest that regulation of AS expression involves interplay among positive transcriptional regulators c-Myc and Sp4, and negative regulator HIF-1α that confers resistance to ADI treatment in A2058 and SK-MEL-2 cells. Inability of AS induction in A375 cells under arginine depletion conditions was correlated by the failure of c-Myc to interact with the AS promoter. [Mol Cancer Ther 2009;8(12):3223–33]

Introduction
The incidence of malignant melanoma continues to increase in the past decade. Conventional immunotherapy-, chemotherapy-based, or combination of these therapies yield an overall response rate of <25%. Thus, novel strategies for treating melanoma have been sought (1, 2). It has been reported that majority of melanomas and hepatocellular carcinomas are auxotrophic for arginine because of their lack of argininosuccinate synthetase (AS) expression (3–5). Arginine is synthesized from citrulline through two-step reactions catalyzed by AS and argininosuccinate lyase, in which AS is the rate-limiting enzyme. In light of these unique properties, arginine deiminase (ADI), a bacterial enzyme that converts arginine to citrulline and ammonium, resulting in arginine deprivation, was developed for treatment of melanoma and hepatocellular carcinomas. To suppress its immunogenicity and enhance its stability in circulation, the clinically used ADI (ADI-PEG20) was conjugated with polyethylene glycol (PEG). In the laboratory setting, exposure of melanoma cells to ADI results in cell death, whereas normal cells that express AS are able to survive (6–9). Phase II clinical trials using ADI-PEG20 has been conducted in melanoma and hepatocellular carcinomas. Arginine was successfully degraded from serum by ADI-PEG20, and a response has been seen (6–9). In some patients, however, insensitivity to ADI treatment was associated with the induced expression of AS (10), suggesting that induction of AS expression under ADI treatment may contribute to failure of ADI treatment. However, the mechanisms by which AS was expressed in ADI-treated patients have not been elucidated.

The current study was initiated to investigate the mechanism by which AS is induced in three melanoma cell lines under arginine deprivation conditions, either by treating these cells with ADI-PEG20 or by culturing them in arginine-free medium. We found that the inducibility of AS expression was correlated with ADI resistance and that expression of AS was reversibly regulated by arginine availability. Further investigations revealed that induction of AS expression by arginine depletion involves the positive transcriptional regulator c-Myc and negative regulator HIF-1α, which recognize the E-box in collaboration with Sp4, which recognizes the GC-box at the AS promoter in A2058 and SK-MEL-2 cells. Failure of AS induction in A375 melanoma
cells was associated with the inability of c-Myc to interact with the E-box. Our study reveals the important roles of c-Myc/HIF-1α/Sp4 in the regulation of AS expression that confer ADI-PEG20 resistance in melanoma cells.

Materials and Methods

Reagent, Cell Culture, and Antibodies

ADI-PEG20 (specific activity, 5–10 IU/mg) was obtained from Polaris Pharmaceutics, Inc. Deferoxamine, cobalt chloride, and sulfurhodamine B (SRB) were purchased from Sigma and LY294002 was purchased from Cayman Chemical.

A2038, SK-MEL-2, and A375 melanoma cells were purchased from American Type Culture Collection Center and maintained in DMEM containing 10% fetal bovine serum. Arginine-free medium was purchased from Invitrogen and was supplemented with 10% fetal bovine serum that had been dialyzed against PBS. For arginine depletion, cells were washed with PBS and maintained in DMEM containing 0.05 μg/mL ADI-PEG20 or in arginine-free medium.

For cell growth assay, 100 to 500 cells were seeded in 96-well plates for various lengths of time. Cells were fixed with 10% tri-chloro acetic acid, washed with distilled water, and stained with 0.4% SRB. The excess SRB dye was washed with 0.1% acetic acid and the remaining SRB was dissolved in 10 mmol/L Tris buffer. The relative number of cell was measured by absorbance at 405 nm.

Mouse anti-β-actin, anti-HA (Sigma), mouse anti-AS (BD Bioscience), mouse anti-myc (9E10, Roche), rabbit anti-Sp1 (Santa Cruz Biotechnology), rabbit anti-Sp2 (Santa Cruz Biotechnology), rabbit anti-Sp3 (Santa Cruz Biotechnology), rabbit anti-Sp4 (Santa Cruz Biotechnology), rabbit anti-c-Myc (N262, Santa Cruz Biotechnology), mouse anti-c-Myc (C33, Santa Cruz Biotechnology), and mouse anti-HIF1α (BD Bioscience) were purchased commercially.

Plasmid DNA Construction and siRNA Transfection

The expression vector for pCGN-HA-c-Myc was generously provided by Dr. Jonathan M. Horowitz, North Carolina State University, Raleigh, NC (12). The Sp3 expression vector was generously provided by Dr. William P. Tansey, Ruttenberg Cancer Research.

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The proximal promoter region of AS gene (from –1822 to +300) was cloned by PCR using GC-rich PCR system (Roche). The used primers are F: 5′-ACCGTCTCTACTGTCCTCGTATCTGACTGG-3′, R: 5′-ACTGAGCGGGGCGCGGGCGCGTCTTACAG-3′. The deletion constructs were created by PCR using the same primer and following forward primers:

-1334: 5′-ACCGTCTCTGTAGAGAGGAGGAGGAGGG-3′
-846: 5′-ACCGTCTCTTGAGGAGGCCGAAGCAGACAGT-3′
-602: 5′-ACGGTGAGGATGAAAGCAGGGGCTCCGTTCCGG-3′
-358: 5′-ACGGTGAGGATGAAAGCAGGGGCTCCGTTCCGG-3′
-85: 5′-ACGGTGAGGATGAAAGCAGGGGCTCCGTTCCGG-3′, and
-37: 5′-ACGGTGAGGATGAAAGCAGGGGCTCCGTTCCGG-3′. The amplified DNA fragments were cloned using TOPO PCR system (Invitrogen) and sequence integrity was analyzed. The promoter region were digested by MluI/Xhol and ligated with pGL3 vector. The point mutations were introduced into E-box and GC-box sequence by using mutagenesis kit (Stratagene).

The siRNAs for HIF1α (5′-GAUUACUCAUGUGUUGAGCU-3′) and AS (5′-GCAUGAAGCAUGGAGUA-3′) were synthesized by Sigma, c-Myc siRNA was purchased from Cell Signaling, and control siRNA and Sp4 siRNA were from Santa Cruz Biotech.

Immunoprecipitation and Immunoblotting

All immunoprecipitation experiments were done as described previously (16). Briefly, protein samples were incubated with an antibody and 25 μL 50% protein A sepharose slurry, and Protein A beads were collected. Immunoprecipitates were resolved by SDS-PAGE and analyzed by immunoblotting. For immunoblot analysis, the protein samples were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked, incubated with primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies, and visualized by an enhanced chemiluminescence kit.

Reverse Transcription-PCR

Total RNA was isolated with trizole reagent (Invitrogen) following manufacturer's instruction. The CDNA was synthesized from 1 μg of total RNA by using a Super Script II system (Invitrogen). The synthesized CDNA was subjected to standard PCR or real-time PCR using following oligonucleotide primers:

c-Myc: forward, 5′- CCTACCCCTCTCAACGA-CAGG-3′ and reverse, 5′-ACTCTGACCTTTCCAGGA-3′; ASS, forward, 5′-AGGCACCACATCTTTACCAGT-3′ and reverse: 5′-CTGACCTTCTCTTCCTCCTC-3′; β-actin, forward: 5′-AGGAGGAAGCAAGAAGAG-3′ and reverse: 5′-AGGAGGCTACAGGGATAAGCA-3′.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation ChIP assay was carried out with a ChIP assay kit (Upstate Biotechnology) following manufacturer's instruction. A GC-rich PCR system (Roche) was used for PCR analysis using following AS promoter–specific primers (forward: 5′-TGGAGTATATGATGGTACCCGCGC-3′, reverse: 5′-GCCCATCCCAGTTAATAACGAAG-3′) or primers for glyceraldehyde-3-phosphate dehydrogenase promoter (forward: 5′-CTGCTGCCGAAATATATG-3′, reverse: 5′-GGAGTGAGAGGAGGATAAGCA-3′). The quantitative analyses on enrichment of precipitated DNA were also done as described previously (17).

Luciferase Assay

Luciferase assay was carried out using dual promoter gene expression reporter kit (Promega) following manufacture's instruction. In brief, cells cultured in 24-well dishes were transfected with 0.2 μg of pGL3 vector and 2 ng of pRLII vector using Lipofectamine 2000 (Invitrogen) following manufacturer's instruction. After incubation in normal medium containing 0.05 μg/mL ADI or in arginine-free medium,
cells were lysed with passive lysis buffer. Cell lysates were cleared by centrifugation and supernatants were used for dual luciferase assay. The obtained luciferase activity was normalized with that of empty pGL3 vector.

Results

Induction of AS Expression by Arginine Depletion Is Associated with ADI Resistance in Melanoma Cell Lines

We first analyzed the effects of ADI treatment on AS expression in A2058 melanoma cells. A2058 cells were maintained in medium containing 0.05 μg/mL ADI for the time intervals as indicated. Thereafter, cells were cultured in normal medium for an additional 24, 48, and 72 h. The cells were harvested and AS expression levels were analyzed by Western blotting or real-time PCR. The AS mRNA levels are represented as the expression level relative to β-actin. A, A2058, SK-MEL-2, and A375 cells were maintained in normal medium, medium containing 0.05 μg/mL ADI, or arginine-free medium for 72 h and AS expression levels were analyzed by Western blotting and real-time PCR. The AS mRNA levels are represented as expression level relative to β-actin. B, proliferation of A2058, SK-MEL-2, and A375 cells after ADI treatment or being cultured in arginine-free medium. Cells (100 each) were seeded on 96-well plates and cultured in normal medium, medium containing 0.05 μg/mL ADI, or arginine-free medium. Cells were fixed by 10% trichloroacetate every 24 h and the growth rate was analyzed by SRB assay. C, A2058 cells were transfected with control or AS siRNA (100 nmol/L each) and maintained in normal medium, medium containing 0.05 μg/mL ADI, or arginine-free medium and cell growth rate was analyzed. Seventy-two hours after transfection of AS siRNA, AS expression levels were examined by Western blotting 72 h after transfection. Points, mean by Student’s t test from three independent experiments (*, P < 0.01); bars, SD.

Figure 1. Induction of AS expression by arginine depletion contributes to ADI resistance. A, kinetic study of AS induction by arginine depletion. A2058 cells were maintained in medium containing 0.05 μg/mL ADI for the time intervals as indicated. Thereafter, cells were cultured in normal medium for an additional 24, 48, and 72 h. The cells were harvested and AS expression levels were analyzed by Western blotting or real-time PCR. The AS mRNA levels are represented as the expression level relative to β-actin. B, A2058, SK-MEL-2, and A375 cells were maintained in normal medium, medium containing 0.05 μg/mL ADI, or arginine-free medium for 72 h and AS expression levels were analyzed by Western blotting and real-time PCR. The AS mRNA levels are represented as expression level relative to β-actin. C, proliferation of A2058, SK-MEL-2, and A375 cells after ADI treatment or being cultured in arginine-free medium. Cells (100 each) were seeded on 96-well plates and cultured in normal medium, medium containing 0.05 μg/mL ADI, or arginine-free medium. Cells were fixed by 10% trichloroacetate every 24 h and the growth rate was analyzed by SRB assay. D, A2058 cells were transfected with control or AS siRNA (100 nmol/L each) and maintained in normal medium, medium containing 0.05 μg/mL ADI, or arginine-free medium and cell growth rate was analyzed. Seventy-two hours after transfection of AS siRNA, AS expression levels were examined by Western blotting 72 h after transfection. Points, mean by Student’s t test from three independent experiments (*, P < 0.01); bars, SD.

We then determined the expression of AS in two other melanoma cell lines, SK-MEL-2 and A375. Like A2058 cells, these cells have intact AS genes in their genomes by sequencing (data not shown). The cells were maintained in ADI-containing or arginine-free medium for 72 hours. AS expression was induced in SK-MEL-2 cells but the level of induction was lower than that in A2058 cells. AS expression was not detected in A375 cells even under induction conditions (Fig. 1B). These three melanoma cell lines were subjected to sensitivity test under arginine-deprivation culturing conditions,
whereas the regular medium (DMEM) we used contained 0.48 mmol/L arginine. A2058, SK-MEL-2, and A375 cells cultured under arginine-free medium showed very little proliferative activity up to 5 d. ADI treatment completely inhibited the growth of A375 cells for the same period of time, but A2058 and Sk-MEL-2 cells showed significant growth in the presence of ADI (Fig. 1C). These data indicate that AS inducibility correlates with ADI resistance. Indeed, we were able to establish ADI-resistant cell lines from A2058 and from SK-MEL-2 cell lines but not from A375 cell lines (data not shown). We also noticed that A2058 and SK-MEL-2 cells could partially maintain growth under ADI treatment but not in the arginine-free medium, yet both culturing conditions can induce AS expression (Fig. 1B). These results suggest that induction of AS alone is not sufficient to support cell growth under complete arginine-deprivation conditions.

To address the role of AS induction in ADI resistance, we used siRNA to knockdown AS mRNA. As shown in Fig. 1D, transfection with AS siRNA effectively suppressed AS induc- tion in ADI-containing medium or arginine-free medium, and there was a concomitant reduction in cell growth, supporting the role of AS expression in ADI resistance.

Figure 2. Analysis of arginine deprivation–responsive cis-elements on the AS promoter. A, deletion analysis of the AS promoter. A2058 cells were transfected with pGL3-AS recombinants encoding various lengths of AS promoter sequences and pRLII. Seventy-two hours after transfection, the promoter activities of each construct were assayed. B, mutation analysis of the AS promoter. The point mutations were introduced on the E-box and/or GC-box of pGL3-AS-85 as indicated. A2058 cells were transfected with mutant AS promoter constructs and promoter activity was assayed. The AS promoter activity was normalized with that of the empty pGL3 vector. Columns, mean by Student’s t test from three independent experiments. (*, P < 0.01); bars, SD. C, E-box–dependent activation of AS promoter activity by c-Myc. A2058 cells were transfected with wild-type, GC-box mutant, E-box mutant, or double mutant of pGL3-AS-85 with empty or c-Myc expression vector and maintained in normal medium, medium containing 0.05 μg/mL ADI, or arginine-free medium for 72 h. The AS promoter activity was analyzed and was normalized with that of the pGL3 vector. Columns, mean from three independent experiments; bars, SD. D, A2058 cells were cotransfected with pGL3-AS-85 and expression vectors for Sp1, Sp2, Sp3, and Sp4. The promoter activity and the expression of transfected vectors were analyzed. Columns, mean from three independent experiments (*, P < 0.01); bars, SD. Expression levels of the transfected (+) Sp1, Sp2, Sp3, and Sp4 were determined by Western blottings using their respective antibodies. Representative expression levels of β-actin were used as loading control. E, additive effect of c-Myc and Sp4 in AS promoter activity. A2058 cells were transfected with pGL3-AS-85 and expression vectors for c-Myc and Sp4 alone, or c-Myc plus Sp4, and AS promoter activity was analyzed. The AS promoter activity was normalized with that of the empty pGL3 vector. Columns, mean from three independent experiments; bars, SD.
Identification of Arginine Deprivation-Responsive cis-Elements in the AS Promoter

To identify the cis-elements within the AS promoter that respond to arginine availability, we first constructed a reporter recombinant pGL3-AS-1822 that contains nucleotides −1822 to +300 of the AS promoter linked to the bacterial luciferase reporter gene. We also constructed several AS promoter deletion reporters by progressively removing the upstream AS sequences in pGL3-AS-1822. These deletion reporters were transfected into A2058 cells and their promoter activity was analyzed. As shown in Fig. 2A, the reporters with deleted sequences down to −85 nucleotide still retained responsiveness to arginine depletion, but further deletion to −37 nucleotide markedly reduced both basal and arginine depletion-responsive activities, suggesting that −85 to −37 nucleotides contain sequence important for both basal and inducible AS expression. By using computational analysis described previously (18), we found an E-box and a GC-box sequences between −85 and −37 nucleotides of the AS promoter (Fig. 2B).

To determine whether these sequences are involved in AS induction, we introduced mutations into both the E-box and GC-box in pGL3-AS-85 (Fig. 2B, underscores) and analyzed their effects on AS promoter activity. Mutations at either the E-box or the GC-box alone reduced AS promoter activity and compound mutations further reduced the promoter activity in response to arginine depletion (Fig. 2B, bottom). These results suggest that both E-box and GC-box sequences play important roles in the induction of AS by arginine depletion.

Two basic helix-loop-helix transcription factors, c-Myc and HIF-1α, were considered as strong candidates for interacting with the E-box sequence. We first analyzed whether c-Myc indeed plays a role in regulating AS expression. Wild-type or mutant pGL3-AS-85 reporter recombinants were cotransfected with empty or c-Myc expression vector. As shown in Fig. 2C, cotransfection with c-Myc expression vector upregulated reporter activity of wild-type and GC-box mutated pGL3-AS-85, but not of the mutant E-box or double mutant constructs. These results indicate that c-Myc positively regulates AS promoter activity through its interaction with the E-box.

The Sp1 transcription factor family is well known to regulate their target genes through interactions with the GC-box. To investigate the impact of Sp1 transcription factors on the AS promoter, we analyzed the effect of cotransfection of expression vectors encoding Sp1, Sp2, Sp3, and Sp4 on AS promoter activity. Cotransfection with Sp4 recombinant showed a 4-fold induction of reporter expression levels, whereas cotransfection with Sp3 recombinant reduced >50% of reporter expression (Fig. 2D). Cotransfection with Sp1 and Sp2 recombinants increased reporter activity by <1.5-fold (Fig. 2D, left). Expression levels of transfected Sp1, Sp2, Sp3, and Sp4 were comparable in these transfections (Fig. 2D, right). These results suggested that Sp3 and Sp4 can modulate AS promoter activity. The role of Sp4 in transcriptional upregulation of AS was also shown by the additive increases in reporter expression when c-Myc and Sp4 expression recombinants were cotransfected (Fig. 2E).

Induction of c-Myc Expression and Its Cross-talk with Sp4 in the Arginine Depletion–Induced AS Expression in A2058 Cells

To address the roles of c-Myc, Sp4, and HIF-1α (see below) in regulating AS expression under the stress of reduced arginine in melanoma cells, we first focused on A2058 cells. We found that levels of c-Myc but not of Sp4 were increased in cells grown in medium-containing ADI or in arginine-free medium. Upregulation of c-Myc was also observed at the mRNA level (data not shown). We then investigated the effects of c-Myc or Sp4 expression on AS induction. A2058

Figure 3. c-Myc is induced by arginine-depletion and positively regulates AS promoter activity through the E-box in A2058 cells. A, cells were transfected with 100 nmol/L of scramble (control) or c-Myc siRNA. Cells were maintained in normal, ADI-containing, or arginine-free medium for 48 h. Expression levels of AS, c-Myc, and β-actin were analyzed by Western blotting. B, cells were transfected with control or Sp4 siRNA and maintained in normal medium, medium containing 0.05 μg/mL ADI, or arginine-free medium for 72 h. Expression levels of AS, Sp4, and β-actin were analyzed by Western blotting. C, the interaction between c-Myc and Sp4 was analyzed by coimmunoprecipitation using anti-c-Myc antibody followed by Western blotting using anti-Sp4 and anti-c-Myc antibodies. D, the interaction between Sp4 and c-Myc was analyzed by coimmunoprecipitation using anti-Sp4 antibody followed by Western blotting using anti-c-Myc and anti-Sp4 antibodies.
cells were transfected with c-Myc siRNA, Sp4 siRNA, or scramble siRNA. Cells were maintained in normal, ADI, or arginine-free medium thereafter for 48 hours. As expected, in the presence of control siRNA, c-Myc but not of Sp4 (Fig. 3B) protein levels were upregulated by arginine depletion. Knockdown of c-Myc by siRNA to <50% in the ADI-treated or arginine-free medium-cultured cells resulted in >80% suppression of AS induction, suggesting that c-Myc is a positive regulator for AS upregulation (Fig. 3A). Knockdown Sp4 to ∼30% of the control level by siRNA also inhibited AS induction (Fig. 3B), suggesting that Sp4 is also involved in AS induction. The extent of AS suppression by Sp4 siRNA seemed to be much reduced in comparison with the effect of c-Myc knockdown (Fig. 3A). However, knockdown of Sp3 levels to ∼50% of control by siRNA did not significantly altered AS expression levels (data not shown).

Figure 4. Roles of HIF-1α in the regulation of AS expression. A, arginine deprivation suppresses HIF-1α expression. A2058 cells were maintained in normal medium, medium containing 0.05 μg/mL ADI, or arginine-free medium with or without 100 μmol/L deferoxamine (DFX) or 100 μmol/L CoCl2. Seventy-two hours after treatment, cells were harvested and subjected for Western blotting. B, A2058 cells were transfected with 100 nmoL scramble (control) or HIF-1α siRNA and maintained in normal medium, medium containing 0.05 μg/mL ADI, or arginine-free medium with 100 μmol/L deferoxamine. Untransfected A2058 cells maintained in normal medium, medium containing 0.05 μg/mL ADI, or arginine-free medium were used for control. Levels of AS, HIF-1α, c-Myc, Sp4, and β-actin were measured by Western blotting. C, A2058, SK-MEL-2, and A375 cells were transfected with c-Myc or cotransfected with HIF-1α expression vector, and pCGN (control) vector, and cell lysates were analyzed by Western blot. As an expression control, the level of total expression of each protein was examined by immunoblotting with antibodies against whole protein as indicated or β-actin (loading control). D and E, kinetics of HIF-1α, AS, c-Myc, and Sp4 expression in A2058 cells grown in arginine-free medium (D) or 0.05 μg/mL ADI-containing medium (E) for the time courses as indicated.
shown). We repeatedly observed that the c-Myc siRNA probe we used was less effective in suppressing its target RNA in A2058 cells grown under regular conditions than in those grown under induced conditions. This was probably due to the low abundance of c-Myc mRNA in cells grown under noninduced conditions, thereby providing reduced target size for the attack by siRNA. Nonetheless, this difference should not affect the result that knockdown of c-Myc in the ADI- or arginine-free medium-treated cells was associated with downregulation of AS expression.

We then explored the possibility of physical interactions between c-Myc and Sp4. c-Myc protein was immunoprecipitated from A2058 cell lysate by anti–c-Myc antibody and the precipitate was subjected to Western blotting using anti–c-Myc and anti-Sp4 antibodies. Figure 3C shows that Sp4 was coimmunoprecipitated with c-Myc from the lysate of cells treated with ADI or cultured in arginine-free medium, but not from cells maintained in normal medium. The interactions between c-Myc and Sp4 were also shown by using anti-Sp4 antibody in reciprocal immunoprecipitation, which showed that c-Myc was coimmunoprecipitated with Sp4 from the lysate of cells treated with ADI or cultured in arginine-free medium but not from cells maintained in the regular medium (Fig. 3D). These results showed the physical interactions between c-Myc and Sp4 in A2058 cells cultured under arginine-depleted conditions.

**HIF-1α Is a Negative Regulator for Arginine Depletion-Induced AS Expression in A2058 Cells**

Arnt/HIF-1β is another potential interacting transcription factor for the E-box sequence. HIF-1α forms heterodimer with HIF-1α and modulates target gene expression under hypoxic condition. Given that the physiologic role of HIF1α is often opposite to that of c-Myc (19–21), we explored the possible involvement of HIF-1α in AS gene expression. Figure 4A shows that treating A2058 cells with ADI or growing them in arginine-free medium resulted in downregulation of HIF-1α (compared among lanes 1–3; results were clearly observed with prolonged exposure of the autoradiograph; data not shown). Induction of HIF-1α using hypoxia mimics CoCl2 or deferoxamine resulted in accumulated HIF-1α levels, and elevated HIF-1α was associated with downregulation of AS expression. Strikingly, downregulation of HIF-1α in the hypoxia mimics–treated A2058 cells by siRNA reversed the inhibitory effects of HIF-1α on AS expression in the arginine-depleted conditions (Fig. 4B). These results showed that HIF-1α plays a negative role in the regulation of AS expression under arginine-depleting conditions. Moreover, we found that in the CoCl2− and deferoxamine-treated A2058 cells, accumulation of HIF-1α in the ADI-treated or arginine deficiency–cultured A2058 cells was associated with downregulation of c-Myc (Fig. 4A, compare lanes 5, 6, 8, and 9 with 2 and 3). The downregulation of HIF-1α by siRNA

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Mechanistic investigation of c-Myc, HIF-1α, and Sp4 in the regulation of AS expression in melanoma cells under arginine-available and arginine-deficient conditions. A, Western blotting analyses of expression levels of AS, HIF-1α, c-Myc, and Sp4 in A2058, SK-MEL-2, and A375 cells grown in the regular, ADI-containing (0.05 μg/mL), and arginine-free medium for 72 h. B to D, ChIP assay of the interactive transcriptional regulators with AS promoters under arginine depletion conditions. B and C, c-Myc displaces HIF-1α binding from AS promoter in response to arginine depletion in A2058 and SK-MEL-2 cells, respectively. D, dissociation of HIF-1α, but no concomitant association of c-Myc, to the AS promoter in response to arginine depletion in A375 cells. E, stabilization of HIF-1α by the hypoxia mimic CoCl2 resulted in persistent association between HIF-1α and the AS promoter and in the prevention of c-Myc to interact with the promoter in A2058 cells cultured under arginine-deficient conditions. Top, the antibodies used for ChIP and control IgG. Input, genomic DNA prior to immunoprecipitation. AS promoter sequences from ChIP were quantified by PCR assay.
abolished the inhibitory effect of deferoxamine on AS induction through the recovery of c-Myc induction (Fig. 4B, comparing lanes 8 and 9 with 5 and 6). These results suggest that HIF-1α also exerts a suppressive role in the regulation of AS induction through downregulation of the c-Myc pathway in A2058 cells.

We next investigated the effects of overexpression of c-Myc and HIF-1α on the expression of AS. We transfected HA-tagged c-Myc recombinant DNA into A2058, SK-MEL-2, and A375 cells. Overexpression of c-Myc induced AS expression in A2058 and SK-MEL-2 cells, but not in A375 cells (Fig. 4C, left). Cotransfection of Myc-tagged HIF-1α with HA-c-Myc recombinants suppressed c-Myc–induced AS expression (Fig. 4C, right). These results support the positive role of c-Myc and negative role of HIF-1α in regulation of AS expression.

To determine the expression kinetics of transcription regulators and the induction of AS, we analyzed the time course of HIF-1α, c-Myc, and Sp4 expression in reference to AS expression in A2058 cells after treatment with ADI or under arginine-free culture conditions. We chose A2058 cells because they express comparably higher steady-state levels of HIF-1α than do SK-MEL-2 and A375 cells (see below; Fig. 5A). With prolonged exposure of autoradiographs, HIF-1α signals in the Western blots could be clearly visualized. Figure 4D and E show that reduction of HIF-1α was seen within 10 minutes after the treatment, whereas induction of c-Myc started 4 hours after the treatment, and rapid surge of AS levels was seen 48 to 72 hours thereafter. Levels of Sp4 did not change throughout the entire time course analysis. These results indicate that induction of AS expression by arginine deprivation in A2058 cells is a delayed effect, initiated by the downregulation of HIF-1α and followed by the upregulation of c-Myc.

Mechanistic Analyses of c-Myc, HIF-1α, and Sp4 in the Regulation of AS Expression in Melanoma Cell Lines in Response to Arginine Deprivation

We then investigated the mechanistic aspects of c-Myc, HIF-1α, and Sp4 in the induced AS expression in A2058, SK-MEL-2, and A375 cells. Expression of HIF-1α, c-Myc, and Sp4 in these three cell lines were determined by Western blotting. Our results showed that: (a) comparing with A2058 cells, the steady-state levels of HIF-1α were much reduced in SK-MEL-2 and A375 cells, regardless of arginine availability (Fig. 5A); (b) like A2058 cells, levels of c-Myc were low in SK-MEL-2 cells under noninduced conditions but were induced in the ADI- and arginine-free medium–treated cells. Furthermore, when induced levels of c-Myc between A2058 cells and SK-MEL-2 cells were compared, it appears that levels of c-Myc were correlated with levels of AS induction. These results are consistent with the positive role of c-Myc in the regulation of AS expression in these two cell lines; (c) levels of c-Myc were high in A375 cells grown in the regular medium but were lower under arginine-depleting conditions; and (d) levels of Sp4 were constitutively expressed in all the three melanoma cell lines, regardless of arginine availability.

To gain mechanistic insights into how these transcription factors regulate AS expression in response to arginine depletion, we carried out ChIP analysis. A2058 cells grown in the regular medium, arginine-free medium, or treated with ADI-PEG20 were fixed with formaldehyde, sonicated, and the sheared chromatin was immunoprecipitated by antibodies against c-Myc, HIF-1α, or Sp4. The precipitated DNA fragments were renatured, purified, and subjected to PCR analyses. Significant enrichment of AS promoter sequence was observed in the chromatin fractions of ADI-treated and arginine-free–cultured cells pull-down by anti-c-Myc antibody. In contrast, reduction of AS promoter sequence was observed in the samples when anti–HIF-1α antibody was used. No significant difference of AS sequence when Sp4 antibody was used (Fig. 5B). These results suggest that when A2058 cells were cultured under the noninduced conditions, the AS promoter was mainly bound by HIF-1α but not by c-Myc. When these cells were switched to ADI–containing or arginine-free medium, downregulation of HIF-1α resulted in the replacement of c-Myc occupancy at the AS promoter. Similar results were observed in SK-MEL-2 cells (Fig. 5C).

We hypothesized that AS expression in A375 cells could not be induced by ADI treatment because c-Myc was unable to participate in transcriptional activation at the AS promoter level. To test this hypothesis, we performed ChIP analyses. As shown in Fig. 5D, although dissociation of HIF-1α from the AS promoter was seen in A375 cells cultured under arginine-depleted conditions, no engagement of c-Myc to the AS promoter was found under these conditions. These results support the hypothesis that failure of induced AS expression in A375 cells under arginine-depleting conditions is because of the inability of c-Myc to interact with AS promoter cultured under arginine-deficient conditions.

We further showed that accumulation of HIF-1α in the CoCl2–treated A2058 cells persists the binding of HIF-1α at the AS promoter and prevents the displacement by c-Myc (Fig. 5E). Alternatively, these results may be explained by that the absence of c-Myc in the engagement AS promoter is due to reduced levels of c-Myc in the CoCl2–treated cells resulting from stabilization of HIF-1α (Fig. 4A). Nonetheless, the present results, at the very least, support that persistent occupancy of AS promoter by HIF-1α is associated with the suppressive effect of AS induction by arginine deprivation, thus supporting the negative role of HIF-1α in the regulation of AS expression.

Discussion

The finding that human malignant melanoma is auxotrophic for arginine provides a molecular basis for the development of targeted therapy of this disease based on arginine deprivation using recombinant ADI (6). In this study, we found that in A2058, SK-MEL-2, and A375 melanoma cell lines, AS expression levels were very low. The expression of AS could be induced in the former two cell lines but not in the last one by ADI. The inducibility of AS expression is correlated with the resistance to ADI. These results have a clinical reminiscence that some melanoma patients do not respond to ADI treatment in clinical trials, underscoring the importance of elucidating the mechanism of induced expression of AS by ADI treatment.
The association between AS expression levels and arginine availability was first noted >40 years ago (22). Multiple mechanisms have been reported for the regulation of AS expression, including transcriptional regulation (23–27) and epigenetic regulation (28, 29). However, many of these studies used nonmelanoma cells that express high basal levels of AS, so the results are not applicable to cancer chemotherapy using ADI because these cells used were not sensitive to ADI treatment. In the present study, we identified an E-box and a GC-box as arginine deprivation–responsive elements that control AS expression and elucidated that c-Myc/HIF-1α are the E-box transcription regulators and Sp4 is the major GC-box factor. Importantly, we describe that c-Myc and HIF-1α function as a positive and a negative regulator, respectively, and that a switch from HIF-1α to c-Myc binding to the E-box is accompanied with the induction of AS expression by arginine deprivation in A2058 and SK-MEL-2 cells (Fig. 6A). We further showed that failure of AS induction in A375 cells by ADI was associated with inability of AS expression under arginine deprivation conditions. A, under the regular culture conditions, HIF-1α is degraded and c-Myc is upregulated and directly binds to the E-box that drives transcription through interaction with the transcription factor Sp4, which is bound to the GC-box of AS promoter. C, in the presence of hypoxia mimics, CoCl2, HIF-1α is stabilized and remains bound at the E-box, thereby preventing the induction of AS expression by arginine depletion. B, failure of c-Myc binding to AS promoter in A375 cells is associated with inability of AS expression under arginine depleting conditions. A, under the regular culture conditions, HIF-1α acts as a repressor by binding to the promoter of AS gene. B, under arginine-depleting conditions, HIF-1α is degraded but c-Myc does not bind to the E-box.

A2058 and SK-MEL-2

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Figure 6. Models depicting induction of AS expression in A2058 and SK-MEL-2 cells by arginine depletion through HIF-1α displacement by c-Myc (A). A, under the regular culture conditions, HIF-1α acts as a repressor by binding to the E-box of AS gene. B, under arginine-depleting conditions, HIF-1α is degraded and c-Myc is upregulated and directly binds to the E-box that drives transcription through interaction with the transcription factor Sp4, which is bound to the GC-box of AS promoter. C, in the presence of hypoxia mimics, CoCl2, HIF-1α is stabilized and remains bound at the E-box, thereby preventing the induced AS expression by arginine depletion. B, failure of c-Myc binding to AS promoter in A375 cells is associated with inability of AS expression under arginine depleting conditions. A, under the regular culture conditions, HIF-1α acts as a repressor by binding to the promoter of AS gene. B, under arginine-depleting conditions, HIF-1α is degraded but c-Myc does not bind to the E-box.

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biogenesis (47). Moreover, it has been reported that disruption of HIF-1α activity using RNAi resulted in increased mitochondrial biogenesis and O2 consumption. Conversely, targeted disruption of c-Myc diminished mitochondrial DNA and O2 consumption (31). These results suggest that HIF-1α and c-Myc are oppositely regulated through mitochondrial metabolism. These observations, together with our finding that HIF-1α can also regulate c-Myc levels, illustrate a complex inter-regulatory network involving HIF-1α and c-Myc through phosphoinositide 3-kinase/Akt signaling in the regulation of AS expression in response to arginine availability. This complex mechanism may explain, at least in part, the observed delayed induction of AS expression by arginine deprivation.

The complex regulatory mechanism of AS expression by arginine deprivation is compounded by the observations that c-Myc–interacting transcription factor Sp4 is also involved. We showed that Sp4 and Sp3 are the GC-box–interacting factors. Previous studies on regulation of AS gene expression have showed that Sp1 interaction with the GC-box is important for transactivation of the AS promoter (23, 24, 48). Our present results show that Sp1 shows only a minor stimulatory function on the AS promoter compared with Sp4 (Fig. 2D). Further investigation using Sp4 siRNA strategy supports the functional role of Sp4 in the regulation of AS expression. Moreover, coimmunoprecipitation results showed a physical interaction between Sp4 and c-Myc. It is important to note that roles of other members of Sp1 family were not examined in these previous studies (23, 24), nor were siRNA and cotransfection assays performed.

This study also raised several important issues that have yet-to-be resolved: (a) Induction of AS expression by arginine deprivation in some melanoma cells is apparently initiated by the rapid disappearance of HIF-1α, because accumulation of HIF-1α by hypoxia mimics blocked the subsequent events leading to suppression of AS induction. It is likely that arginine deprivation induces HIF-1α degradation because HIF-1α mRNA levels were not correspondingly reduced. HIF-1α is a highly unstable protein (t1/2, ~10 minutes under normoxia conditions). The underlying mechanisms that accelerate HIF-1α degradation under arginine-depleted conditions remain to be determined. Alternatively, arginine deprivation may also suppress the translation of HIF-1α. (b) Our results showed that apparently, dissociation of HIF-1α from the E-box is not sufficient and that c-Myc has to be engaged in transcriptional activation of AS expression as shown in A375 cells. The precise mechanism(s) by which c-Myc fails to interact with the AS promoter in A375 cells under arginine depletion conditions are currently unknown. It is plausible that the E-box in A375 chromatin may not be in open configuration due to an epigenetic mechanism such as DNA methylation (29) or masked by yet-to-be determined transcriptional repressors. Alternatively, c-Myc in A375 cells may be sequestered, rendering it unable to transcriptionally activate AS expression under arginine deprivation conditions. (c) The regulatory mechanisms underlying the delayed AS induction by arginine deprivation remain to be critically investigated. (d) Finally, the roles of HIF-1α, c-Myc, and Sp4 in the regulation of AS expression that associated with ADI sensitivity in malignant melanoma chemotherapy remain to be determined. Studies aimed at addressing these issues are currently under way.

In summary, we have revealed the roles of three transcriptional regulators, c-Myc, HIF-1α, and Sp4, in the regulation of AS expression in response to arginine deprivation in three melanoma cell lines. Among which, the accessibility of c-Myc to interact with the AS promoter is critical for the induction of AS expression. Lack of AS expression has been considered a biomarker for sensitivity to ADI treatment in melanomas. Our present study shows that inducibility of AS expression is associated with sensitivity to ADI treatment. These results suggest that mere screening of AS expression levels in melanoma patients before ADI treatment is not sufficient. Our present study has gone one step further and elucidated the mechanisms of AS induction. The identification of positive and negative regulators should provide an important rationale for the development of effective treatment modalities for malignant melanoma through modulation of AS expression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
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References


Molecular Cancer Therapeutics

Resistance to arginine deiminase treatment in melanoma cells is associated with induced argininosuccinate synthetase expression involving c-Myc/HIF-1α/Sp4

Wen-Bin Tsai, Isamu Aiba, Soo-yong Lee, et al.


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