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Stable siRNA-mediated silencing of antizyme inhibitor: regulation of ornithine decarboxylase activity

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Abstract

Ornithine decarboxylase (ODC) is the rate-limiting enzyme involved in the biosynthesis of polyamines essential for cell growth and differentiation. Aberrant upregulation of ODC, however, is widely believed to be a contributing factor in tumorigenesis. Antizyme is a major regulator of ODC, inhibiting ODC activity through the formation of complexes and facilitating degradation of ODC by the 26S proteasome. Moreover, the antizyme inhibitor (AZI) serves as another factor in regulating ODC, by binding to antizyme and releasing ODC from ODC-antizyme complexes. In our previous report, we observed elevated AZI expression in tumor specimens. Therefore, to evaluate the role of AZI in regulating ODC activity in tumors, we successfully down-regulated AZI expression using RNA interference technology in A549 lung cancer cells expressing high levels of AZI. Two AZI siRNAs, which were capable to generate a hairpin dsRNA loop targeting AZI, could successively decrease the expression of AZI. Using biological assays, antizyme activity increased in AZI-siRNA-transfected cells, and ODC levels and activity were reduced as well. Moreover, silencing AZI expression decreased intracellular polyamine levels, reduced cell proliferation, and prolonged population doubling time. Our results directly demonstrate that downregulation of AZI regulates ODC activity, intracellular polyamine levels, and cell growth through regulating antizyme activity. This study also suggests that highly expressed AZI may be partly responsible for increased ODC activity and cellular transformation.

Keywords: Antizyme inhibitor; Ornithine decarboxylase; Antizyme; siRNA

Ornithine decarboxylase (ODC) is a key enzyme in polyamine biosynthesis. Polyamines such as putrescine, spermine, and spermidine are essential for cell growth and differentiation [1]. Therefore, mechanisms of synthesis, catabolism, and transport are highly regulated. Although polyamines are essential for normal cell growth, increased activity of ODC beyond an undefined minimum threshold can induce cell transformation and tumor formation [2]. Elevated ODC activity has been reported in transformed cell lines [3], in virtually all animal tumors including the stomach, skin, colon, esophagus, colorectal [4–7], and in certain tissues predisposed to carcinogenesis [8,9]. Several studies have also demonstrated that upregulation of ODC and polyamine accumulation are necessary for the development of tumor model. Experimentally, induced ODC activity has been observed in transformation caused by oncogenic ras [10], v-Src [11], activated RhoA [12], and overexpression of eukaryotic initiation factor 4E [13], and the c-myc protooncogene can also upregulate ODC transcription [14].

Given the absolute requirement of polyamines for cell growth and the potentially oncogenic consequences of their overproduction, ODC activity and polyamine levels are subjected to tight regulation. Control of mammalian ODC is extremely complex, in part because ODC regulation is both cell-type and stimulus-specific [15–17]. Activities associated with ODC are not only regulated by

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various growth stimuli, but also by polyamines through gene transcription [18], mRNA degradation [19], mRNA translation [20], and enzymatic degradation [21].

Antizyme is a specific inhibitor of ODC, whose synthesis is stimulated by translational frameshifting in response to increased cellular polyamine content [22,23]. Functionally, antizyme binds to the free ODC monomer and prevents the formation of the enzymatically active homodimer. The binding of antizyme to the ODC monomer stimulates the degradation of ODC by the 26S proteasome, thereby reducing ODC level and activity [24]. In addition to inhibiting polyamine biosynthesis, antizyme has also been recently reported to suppress the polyamine transporter [25]. Thus, in a negative feedback system, antizyme plays a pivotal role in the regulation of ODC to prevent excess accumulation of cellular polyamines.

The antizyme inhibitor (AZI) is also involved in the regulation of ODC. AZI stabilizes the ODC level by trapping antizyme. AZI binds to the antizyme with higher affinity than ODC and releases it from the ODC-antizyme complex, preventing ODC from being degraded [26]. Rat and human cDNAs encoding AZI have been cloned and sequenced [26,27], and results show that AZI closely resembles ODC, except that AZI homodimers lack ODC activity. The AZI and ODC monomers do not form heterodimers.

Previously, we found that AZI is highly expressed in tumor tissues compared with counterpart normal tissues [28]. Therefore, it is proposed that enhanced expression of AZI may be responsible for the upregulation in ODC activity in cancer cells. To elucidate the mechanism responsible for AZI regulation of ODC in cancerous cells, we successfully used a recent technological breakthrough that allows delivery of short dsRNA, specifically small interfering RNA, via a plasmid into eucaryotic cells to modulate AZI expression. Using this system, antizyme activity, ODC level and activity, polyamine content, and cell growth in AZI-siRNA-transfected cells were determined. Our study results demonstrate that downregulation of AZI increases antizyme activity, resulting in reduced ODC level and activity, reduced intracellular polyamine content, and eventually leading to decreased cell proliferation.

Materials and methods

Cell culture. The A549 human lung cancer cell line obtained from ATCC was cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (Life Technologies, Gaithersburg, MD) and 1% penicillin/streptomycin (Life Technologies) in a humidified atmosphere containing 5% CO_2 at 37 °C.

SiRNA construction and transfection. To design specific siRNAs targeting AZI, DNA sequences of the type AA(N19) were selected using siRNA Target Finder (www.ambion.com). Four DNA sequences were selected for candidate siRNAs targeting AZI. Scrambled siRNA, 5′-AAACTCTTACGGTCACCAA-3′, was also used as negative control; it does not match any mammalian sequences currently available on online databases. Sense and antisense oligonucleotides were annealed and inserted into the *Bam*HI and *Hin*dIII sites of the pSILENCER 2.1-U6-neo plasmid vector (Ambion, St. Louis, MO), which is a U6 promoter plasmids coding for RNAs composed of two identical 19-nucleotide sequence motifs in an inverted orientation, separated by a 9 base pair spacer to generate a hairpin dsRNA loop capable of mediating AZI inhibition (Fig. 1). A549 cells were transfected using the lipofectamine plus transfection reagent (Invitrogen, Carlsbad, CA) with AZI-siRNAs containing plasmids, scrambled siRNA containing plasmid, and pSILENCER vector control containing no siRNA. Stably expressed clones were selected based on G418 antibiotic resistance (700 μg/mL).

RT-PCR. Total RNA was isolated from transfected A549 cells using Trizol reagent (Life Technologies, Grand Island, NY) and then treated with RNase-free DNase I (Promega, Madison, WI). To detect the presence of AZI in transfected cells, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using primers specific for AZI, forward: 5′-TATGACTTTCGGCTTTGT-3′, reverse: 5′-TCTGGTCC CAAATAGCTA-3′. For detecting expression of β-actin, antizyme, and ODC, the following specific primers were used: β-actin, forward: 5′-GT GGGGCGCCCCAGGCACCA-3′, reverse: 5′-CTCCTTAATGTCA CGCACGATTTC-3; antizyme: forward: 5′-CCTCCACTGCTGTAG TAACCCG-3′, reverse: 5′-CCAAAAAGCTGAAGGTTCGGA-3′; and ODC, forward: 5′-AAAGCAAAGTTGGTTTTGCGG-3′, reverse: 5′-CCTCTGGAAGCCATTGAACGT-3′.

Western blot analysis. Transfected cells were harvested, pelleted by centrifugation, washed with ice-cold phosphate-buffered saline (PBS),

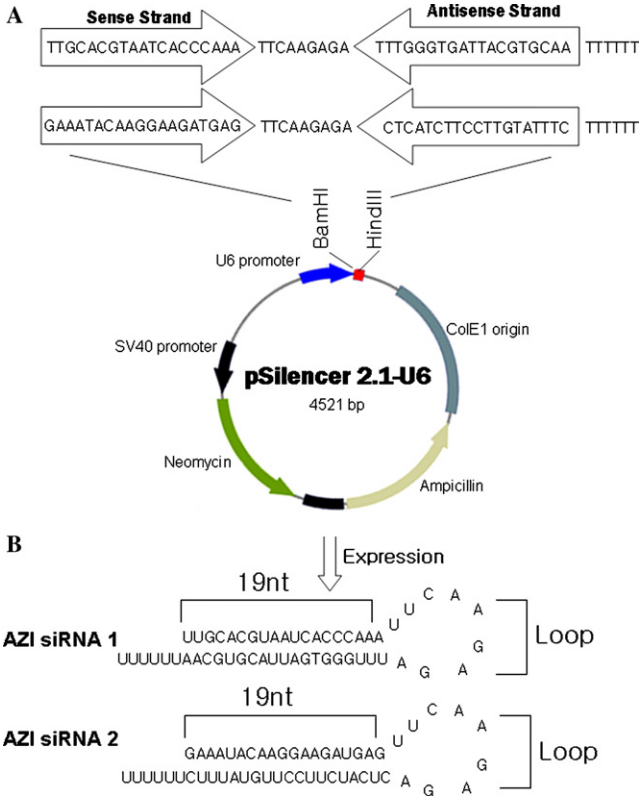

Fig. 1. (A) Schematic representation of the U6 RNA polymerase III promoter-based siRNA expression vector. Sequences encoding siRNA are inserted immediately downstream of the U6 promoter. The 6 thymidine residues serve as the termination signal for polymerase III. (B) Predicted second structure of the AZI-siRNA transcripts from the expression vector.

and lysed in lysis buffer containing 1% Nonidet P-40, 250 mM NaCl, 50 mM Hepes (pH 7.4), 1 mM EDTA, and protease inhibitor. Proteins were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Life Technologies, Gaithersburg, MD). Blots were then probed with polyclonal anti-rabbit AZ (1:1000, kindly provided by Dr. J. Mitchell), monoclonal anti-mouse ODC (1:1000, Sigma, St. Louis, MO), or polyclonal anti-goat actin (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were visualized using the ECL-Plus enhanced chemiluminescence detection system (Santa Cruz Biotechnology).

Assay for ODC and antizyme activity. To measure ODC activity, transfected cells were washed with PBS, resuspended in ODC reaction buffer (10 mM Tris, pH 7.0, 2.5 mM dithiothreitol, 0.1 mM EDTA, and 1% NP-40), and then lysed. The cell lysate was centrifuged at 13,000 rpm for 30 min, and the supernatant was assayed for ODC activity as described previously [29]. Enzyme activity was expressed as cpm/mg protein/h. To assay antizyme activity, transfected cells were pelleted via centrifugation and then homogenized briefly by sonication in 0.02 M potassium phosphate buffer (pH 7.0) containing 2.0 mM dithiothreitol and 0.2 mM EDTA. The cell homogenate (200 μg) was mixed with 200 μg ODC solution produced from A549 cells in 0.1 mL of 0.02 M 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid buffer (pH 7.2) containing 0.5 mM EDTA, 1.0 mM dithiothreitol, and 0.02% Brij-35. After being placed on ice for 15 min, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid buffer was added for a total volume of 0.175 mL, and the mixture was assayed for ODC activity as described above. Antizyme activity was determined by measuring the loss of ODC activity.

Cell proliferation assay. Measuring cell growth, cell counts were performed every 6 h after plating 20,000 transfected A549 cells per well on a 24-well plate. Cells were collected and counted in a hematocytometer using trypan blue exclusion to identify viable cells. Results were plotted on a log scale against a linear time scale. Further, for measuring DNA synthesis, 30,000 transfected A549 cells per well were incubated with [^{3}H]thymidine (1 μCi/well) for 16 h before β-scintillation counting was performed. Cell growth between 48 and 96 h was used to determined doubling time according to the following formula: Doubling time $= [(T - T_0)\log 2]/[\log N - \log N_0]$, where T is the time after inoculation of the cells and N is the number of cells/mm^2.

Polyamine assays. Polyamine content from transfected cells was measured according to the dansylation procedure as described previously [30] using 1,6-diaminohexane (DAH) as the internal standard. Briefly, aliquots (300 μg) of the perchloric supernatants were reacted with 4 volumes of dansyl chloride in acetone (5 mg/mL) in the presence of 50 μL of a saturated solution of sodium carbonate. Excess dansyl chloride was neutralized by reaction with proline (1.8 mg/mL). Then, high performance liquid chromatography (HPLC) separation was performed using a Beckman Ultrasphere ODS 5 μm column (4.6 mm I.D., 25 cm in length). The mobile phase for elution was a linear gradient between 30% acetonitrile in water (V/V, eluent A) and acetonitrile (100%) at a flow rate of 1 mL/min. Fluorescence intensity (excitation at 333 nm and emission at 445 nm) was detected with an Agilent FLD detector and peak intensity was calculated using the Hewlett Packard Chemstation Bioanalytical Software (Laborgeräteborse GmbH, Burladingen, Germany). Under these conditions, bidansyl-putrescine, bidansyl-diaminohexane, tridansyl-spermidine, and tetradansyl-spermine were eluted at 13.78, 14.66, 16.35, and 17.88 min, respectively.

Results

SiRNA mediates down-regulation of AZI transcription

Previous study using the differential display-polymerase chain reaction (DD-PCR) technique to identify genes that are differentially expressed in paired normal and tumor gastric tissues revealed that the ODC antizyme inhibitor, AZI, was highly expressed in human tumor tissue [28]. AZI is known to be involved in the regulation of ODC by trapping the antizyme. Therefore, the upregulation of ODC observed in virtually all cancer forms may be partly ascribed to highly expressed AZI. To elucidate the mechanism of ODC regulation by AZI in cancerous cells, RNA interference was performed to eliminate the expression of endogenous AZI with siRNAs specific for AZI. Four AZI siRNAs were designed using siRNA Target Finder and were stably transfected in A549 cells using pSILENCER 2.1-U6 plasmid vector. To investigate whether designed AZI-siRNAs reduce transcription of the cognate gene in stably transfected A549 cells, AZI mRNA expression was examined in stable transfectants by RT-PCR. AZI-siRNA1, 5′-AATTGCACGTAATCA CCCAAA-3′, that corresponded to nucleotide 421–441, and AZI-siRNA2, 5′-AAGAAATACAAGGAAGATG AG-3′, that corresponded to nucleotide 1018–1038 from the open reading frame of the human AZI mRNA (Fig. 1), could successfully reduce transcription of AZI mRNA, whereas scrambled siRNA failed to reduce the level of AZI mRNA (Fig. 2). The AZI-siRNA1 silenced AZI expression more than AZI-siRNA2. Additionally, as the cDNAs of both ODC and antizyme show extensive homology with AZI, we also measured the transcriptional expression of antizyme and ODC in AZI-siRNA-transfectants. Results showed that AZI-siRNA1 and 2 did not affect transcription of either antizyme or ODC mRNA (Fig. 2). Collectively, these results indicate that transfection of cancerous A549 cells with AZI-siRNA1 and 2 silenced transcription of AZI effectively and specifically.

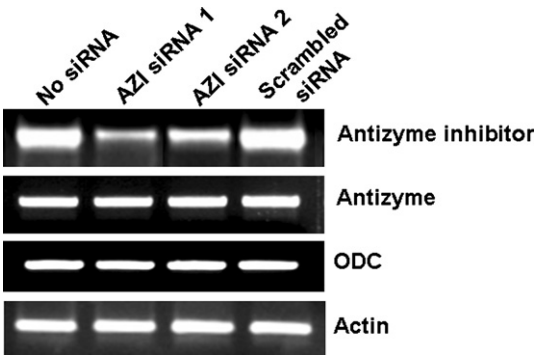

Fig. 2. Quantitative analysis of AZI mRNA in AZI-siRNA-transfected cells. RNA samples were isolated from AZI-siRNA–transfected cells and transfected control cells, and then subjected to RT-PCR using specific primers.

Down-regulation of AZI increases antizyme activity, not protein levels

Since AZI binds to antizyme and inhibits antizyme activity, the effects of AZI-siRNAs on the level and activity of antizyme were investigated. As shown in

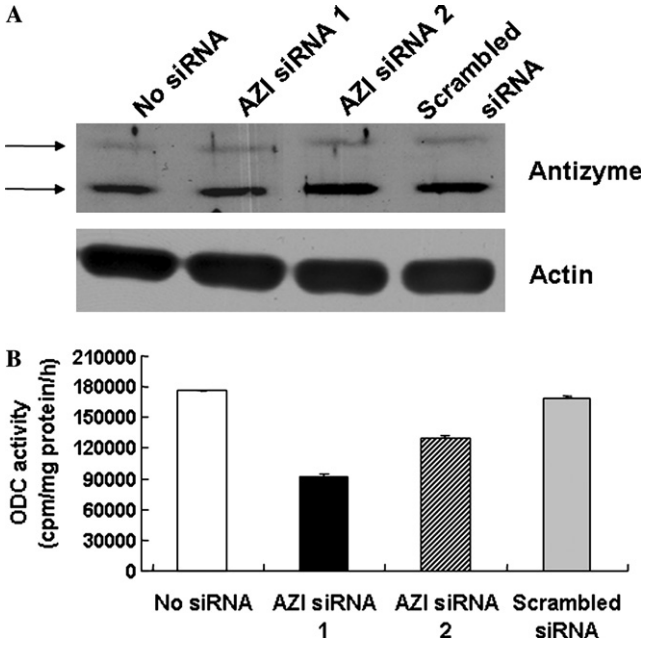

Fig. 3. Effect of siRNA-mediated AZI silencing on antizyme level and activity. After transfectants were cultured for 24 h, whole cell extracts were subjected to (A) Western blot analysis and (B) antizyme activity assay. Data bars represent mean values ± SD, $n = 4$.

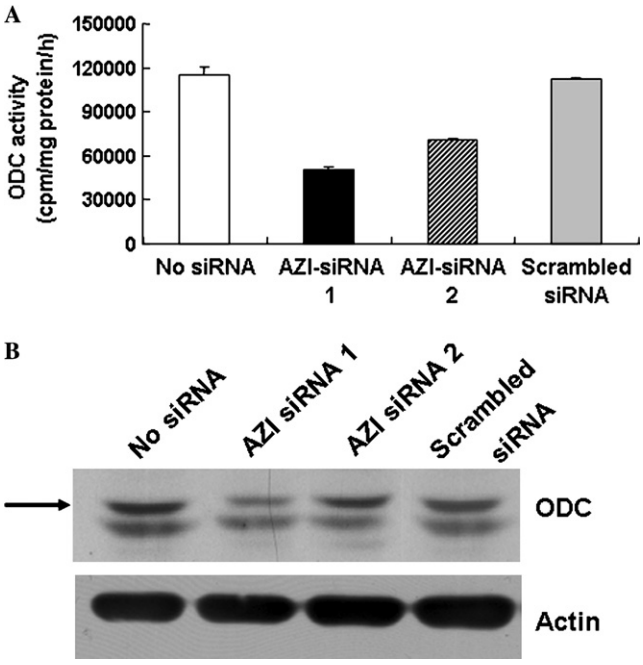

Fig. 4. Effect of siRNA-mediated AZI silencing on ODC activity and ODC levels. After transfectants were cultured for 24 h, whole cell extracts were subjected to (A) ODC activity assay and (B) Western blot analysis. Data bars represent mean values ± SD, $n = 4$.

Fig. 3A, antizyme protein expression was consistent across all groups of differentially transfected cells. However, antizyme activity was increased in AZI-siRNA transfectants compared to the vector-controlled and scrambled siRNA transfectants (Fig. 3B). The increase of antizyme activity was proportional to the amounts of AZI mRNA attenuated by AZI siRNAs. These results demonstrate that AZI directly regulates antizyme activity in A549 cancer cells independent of changes in the antizyme protein expression level.

Down-regulation of AZI expression impairs ODC activity and decreases intracellular polyamine, spermine, and spermidine levels

Antizyme binds to the ODC monomer and stimulates the degradation of ODC through the 26S proteasome, thereby reducing ODC activity. Therefore, we investigated whether down-regulation of AZI affects the level and activity of ODC. As shown in Fig. 4A, ODC activity decreased by 50% and 30% in AZI-siRNA 1 and 2 transfectants, respectively, compared to vector-controlled and scrambled siRNA transfectants. Furthermore, ODC levels were also reduced in AZI-siRNA transfectants, consistent with decreased ODC activity (Fig. 4B), although ODC level in AZI-siRNA2 transfectants was shown to be less reduced. Collectively, these data demonstrate that inhibition of AZI decreased ODC level and activity, which may have resulted from increased antizyme activity after the attenuation of AZI with AZI-siRNAs. In addition, through HPLC analysis, we also determined intracellular polyamine levels in AZI-siRNA transfectants because ODC catalyzes the biosynthesis of polyamine as well as antizyme suppresses cellular uptake of polyamines. As shown in Figs. 5A and B, intracellular polyamines, especially spermidine and spermine, were markedly reduced in AZI-siRNA1 and 2 transfected cells compared with vector-controlled and scrambled siRNA transfected cells. In this condition, putrescine was not detected. These results indicate that down-regulation of AZI expression reduces intracellular polyamine levels in cancerous cells.

Down-regulation of AZI expression suppresses cell proliferation

Polyamines are known to facilitate cell growth. Therefore, this investigation sought to determine whether AZI-siRNA transfection on polyamine content affects cell growth after 96 h. As shown in Fig. 6A, cell growth was reduced in AZI-siRNA1 and 2 transfected cells as compared with vector-controlled and scrambled siRNA-transfected cells. Furthermore, transfection with AZI-siRNAs markedly inhibited DNA synthesis, as demonstrated with results from the [^{3}H]thymidine incorporation assay (Fig. 6B). To determine more precisely the effect of down-regulated AZI expression on cell proliferation, the doubling times of all groups of transfectants were measured. As shown in Table 1, the doubling time of siRNA-AZI 1 and 2 transfectants was 17.8 ± 0.69 and 16.9 ± 0.11 h, respectively, whereas those of vector-controlled transfectants and scrambled siRNA-transfectants

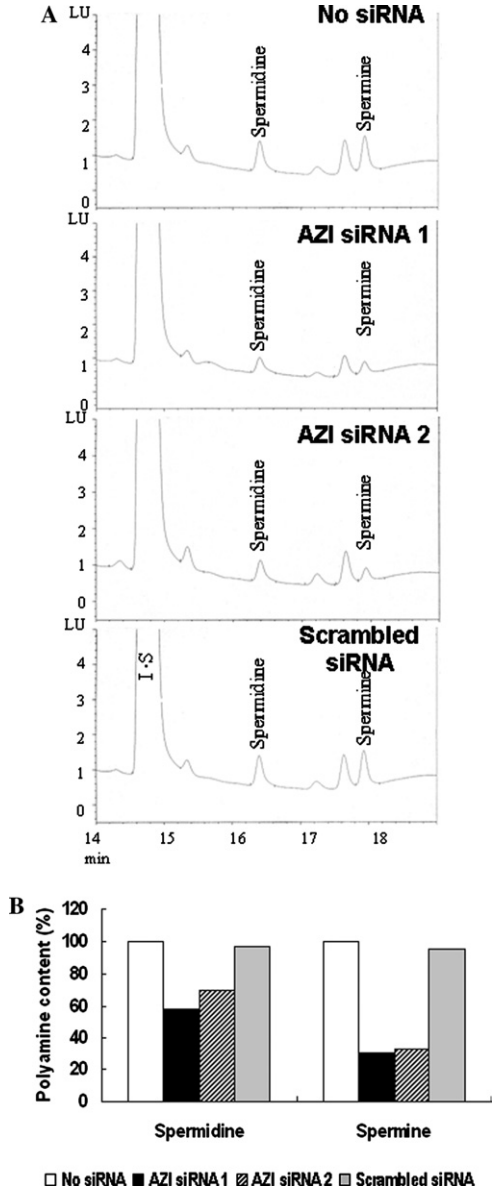

Fig. 5. Effect of siRNA-mediated AZI silencing on intracellular polyamine contents. (A) Representative chromatograms of derivatized polyamines from AZI-siRNA-transfected cells and control transfected cells. (B) Relative quantity of polyamines in AZI siRNA transfectants as compared with control and scrambled siRNA transfectant. Data are representative of one of three independent experiments with comparable results.

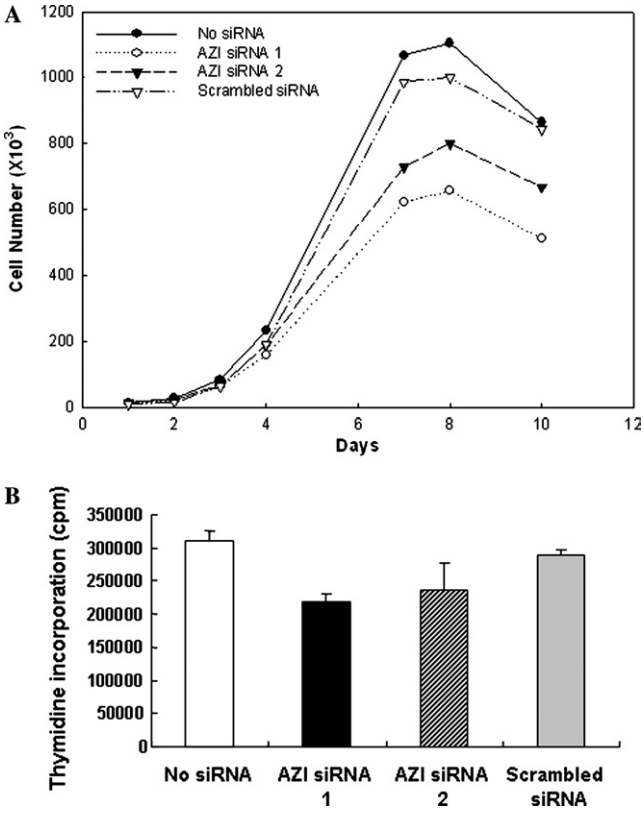

Fig. 6. Effect of siRNA-mediated AZI silencing on cell growth and thymidine incorporation. (A) Transfectants were cultured for up to 96 h. Viable cells were counted at the indicated times. (B) Transfected cells (30,000 cells/well on 24-well plate) were incubated with [^{3}H]thymidine (1 μCi/well) for 16 h, and then β-scintillation counting was performed. Data points or data bars represent mean values ± SD, $n = 4$.

Table 1
The effect of siRNA-mediated AZI silencing on cellular doubling times

Clones	Doubling time (h)
Vector-controlled transfectants	16.3 ± 0.38
AZI-siRNA 1 transfectants	17.8 ± 0.69
AZI-siRNA 2 transfectants	16.9 ± 0.11
Scrambled siRNA transfectants	16.2 ± 0.05

Values represent means ± SD, $n = 4$.

were 16.3 ± 0.38 and 16.2 ± 0.05 h, respectively. The results show that the doubling time of AZI-siRNA transfectants is slightly longer than those of both control transfectants. This may be due to decreased ODC activity and intracellular polyamine levels.

Discussion

Previously we showed that AZI expression was up-regulated in human tumor tissue compared to normal tissue [28]. Therefore, it is hypothesized in this investigation that elevated AZI expression in cancerous cells may lead to an up-regulation of ODC activity by interference with antizyme activity. To determine whether AZI plays an important role in the regulation of ODC in a human lung cancer cell line, we utilized an RNA interference technique with siRNA, and examined changes in the expression levels and activities of antizyme and ODC, as well as intracellular polyamine levels and cell growth in cells stably transfected with siRNA-AZI. In A549 cells stably transfected with AZI-siRNA1 and 2, expression of AZI mRNA was markedly down-regulated. Down-regulation of AZI significantly increased antizyme activity, and consequently

decreased the level and activity of ODC. Finally, suppression of endogenous AZI resulted in a reduction of intracellular polyamine levels, inhibition of cell proliferation, and prolonged doubling time. All those changes were proportional to attenuated amount of AZI mRNA by siRNAs. Collectively, these data clearly indicate that siRNA-induced silencing of AZI mRNA regulates ODC activity by increasing antizyme activity, and therefore changing the phenotype cell growth in A549 cancerous cells.

Accumulating evidence indicates that alterations in ODC regulation and subsequent polyamine accumulation are intimately associated with neoplastic transformation. In the transgenic mouse line carrying ODC cDNA, the frequency of tumor formation increased relative to control mice [31], and the overproduction of ODC was observed to lead to the transformed cell phenotype. Furthermore, elevated ODC activity has reportedly been found in virtually all human cancers. Therefore, cellular ODC activity and polyamine levels are subjected to tight regulation. Several unique features in the molecular mechanism underlying ODC regulation have been documented. The most important ODC regulatory mechanism involves the ODC/polyamine/antizyme negative feedback circuit. Excess polyamine stimulates ODC degradation by promoting biosynthesis of antizymes via enhancement of the efficiency of programmed, ribosomal frameshifts. In addition to its role in polyamine-regulated ODC degradation, antizymes can suppress cellular uptake of polyamines, independent of ODC. Due to this dual negative feedback system, antizyme is very effective in limiting intracellular polyamine levels. However, in mammalian cells, adjustments in antizyme activity are exerted by AZI, a protein without enzymatic activity that is the product of an ODC-related gene. AZI has greater affinity for ODC than ODC has for binding to antizyme, and releases ODC from the ODC-antizyme complex. Functionally, after a growth stimulus instigates a phase of increased ODC production, AZI stabilizes the level of newly synthesized ODC. Several reports have demonstrated that AZI counteracted antizyme-mediated inhibition of ODC activity in vitro [31–33]. The degradation of ODC in reticulocyte lysates was inhibited by the exogenous addition of AZI, which restored ODC activity [29]. Purified recombinant AZI proteins have also been shown to release enzymatically active ODC from antizyme suppression in vitro [32]. Moreover, in zebrafish, the activity of ODC was inhibited by short- and long-antizyme, but zebrafish recombinant AZI reversed the inhibition [33]. However, no existing evidence has directly demonstrated that AZI affects cellular antizyme activity in ODC regulation. In this investigation, we directly show that attenuation in AZI expression level, using two siRNAs targeting AZI in A549 human lung cancer cells, reduces ODC level and activity, as well as decreases intracellular polyamine content via enhancing antizyme activity. Subsequently, cell proliferation is reduced and population doubling time is prolonged. Despite evidence demonstrating the functional role of AZI in the regulation of ODC in cancer cells, and hence cell growth, it cannot be excluded that changes in antizyme and AZI can alter cell growth independent of polyamine levels. Reportedly, antizyme can significantly increase demethylation of 5-methyl cytosine (mC) on CCGG sites via the ODC/polyamine/dc-AdoMet pathway, and suppress malignant phenotypes [34]. Conceivably then, an increase in antizyme activity has growth-inhibitory activities independent of polyamine depletion and that AZI may have other growth-promoting activities, which can completely bypass the putative polyamine effect. Therefore, further experiments are required to demonstrate that changes in polyamine levels are indeed responsible for changes in observed cell generation times.

In conclusion, our results indicate that increased ODC activity in cancer cells partly results from the sequestration of antizyme and subsequent rescue of ODC from degradation, via enhanced AZI expression. From our investigation, the use of AZI-siRNAs effectively reduced AZI expression, which subsequently enhanced antizyme activity, resulting in decreased ODC activity and polyamine content in a human lung cancer cell line. Although our data demonstrated that AZI exerts an indirect effect on cell growth, AZI appears to play an important role in carcinogenesis. Collectively, these results suggest that regulating AZI may be one potential therapeutic target to be used in cancer therapy. However, as the exact regulatory mechanisms involved in AZI expression in human cancers remain unknown at this time, it is essential that further study be completed.

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