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Advances in Brief

Overproduction of Ornithine Decarboxylase Caused by Relief of Translational Repression Is Associated with Neoplastic Transformation[1]

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Abstract

The mRNAs for two key enzymes in polyamine biosynthesis, ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC), both have long 5′ untranslated regions (5′UTRs) that could be important in the regulation of enzyme levels by affecting the translation of these mRNAs. In order to test this hypothesis, ODC and AdoMetDC activities were measured in 3T3 cells and in 3T3 cells overexpressing eIF-4E (P2 cells). eIF-4E has been reported to be a limiting factor in the translation of mRNAs with extensive secondary structures in the 5′UTR. AdoMetDC activity was not greatly different in the two cell lines, but ODC activity was much greater in the P2 cells. These results were confirmed by transfecting these cells with plasmids containing a luciferase complementary DNA fused to follow the 5′UTR from ODC or AdoMetDC. The ODC 5′UTR construct produced a higher luciferase activity in the P2 cells. The high level of expression of ODC may be a critical factor in the transformed phenotype of the P2 cells since the ability of these cells to grow in soft agar was blocked by levels of the ODC inhibitor, α-difluoromethylornithine, that reduced the ODC activity to values comparable to those of the parent 3T3 cells. These results provide more evidence for a critical role of ODC activity in neoplastic transformation and for the importance of its translational regulation in cell growth and transformation.

Introduction

ODC[3] and AdoMetDC are the rate-limiting enzymes in polyamine biosynthesis. Polyamines play an essential role in cell growth and differentiation, and the polyamine biosynthetic pathway may be an important target for the design of chemotherapeutic agents. The cellular activities of the polyamine metabolizing enzymes are tightly controlled. Extensive alterations in enzyme activities can occur very quickly and are usually the result of changes in the absolute amount of enzyme protein (reviewed in Ref. 1). Regulation of these enzymes has been shown to occur at both the transcriptional and translational levels (Refs. 1–8 and references therein). One feature of both ODC and AdoMetDC that may be involved in translational regulation is the unusually long 5′UTRs (>300 bases) of their mRNAs. Although most genes encode mRNAs with relatively short, unstructured 5′UTRs, oncogenes and genes involved in cellular proliferation frequently encode mRNAs with long 5′UTRs that are predicted to form extensive secondary structure (9). An increased ODC activity has been reported to occur in many cells exposed to chemical carcinogens or tumor promoters (1, 8), and prevention of this rise in ODC by use of specific inhibitors such as DFMO reduces tumor formation (reviewed in Refs. 1 and 4). Recently, it has been postulated that the gene coding for ODC may act as an oncogene since expression of ODC at very high levels from appropriate plasmid vectors leads to a transformed phenotype (10, 11).

One way in which the translation of mRNAs with extensive secondary structure might be regulated is by the activation of translation initiation factors that melt mRNA secondary structure. Insulin, which induces ODC activity, also stimulates the phosphorylation of eukaryotic initiation factors eIF-4B and eIF-4E, which are both involved in mRNA binding to the 48S initiation complex, usually the rate-limiting step in translation initiation (12). Phosphorylation of eIF-4B and eIF-4E is increased by various mitogenic stimuli, and it has been suggested that eIF-4E, the least abundant of the translation initiation factors, plays a regulatory role in translation. The importance of eIF-4E in growth control has been demonstrated by Lazaris-Karatzas *et al.* (13), who showed that cells overexpressing eIF-4E exhibit characteristics of cellular transformation, including formation of transformed foci on a monolayer of cells, anchorage-independent growth, and tumor formation in nude mice. Also, synthetic mRNAs containing extensive secondary structure in their 5′UTRs were translated more efficiently in cells overexpressing wild-type eIF-4E than in cells expressing eIF-4E in which serine 53, the major phosphorylation site, has been mutated to an alanine, creating an inactive protein (14). It has been postulated that cell transformation brought about by eIF-4E overexpression may be caused by relief of suppression of translation of mRNAs encoding proteins that regulate cell growth. The experiments described here examined whether ODC and AdoMetDC may be involved in this process and indicate that ODC levels are controlled by eIF-4E and that ODC is an essential component of the transformation of 3T3 cells by this factor.

Materials and Methods

Materials. The NIH-3T3 cell lines used in these studies, including clone 3T3 pMV7–4E(P2) which overexpresses eIF-4E (13, 14), were generous gifts of Dr. N. Sonenberg (McGill University, Montreal, Quebec, Canada). *S*-Adenosyl[*carboxy*-^{14}C]methionine (50 mCi/mmol) and L-[^{14}C]ornithine (52 mCi/mmol) were from DuPont-New England Nuclear (Boston, MA). DFMO was from Marion Merrell Dow Research Institute (Cincinnati, OH). Restriction enzymes were from Stratagene (La Jolla, CA), Promega Corp. (Madison, WI), and Bethesda Research Laboratories (Bethesda, MD). Qiagen plasmid purification columns were purchased from Qiagen, Inc. (Chatsworth, CA). Other biochemical reagents used were purchased from Sigma Chemical Co. (St. Louis, MO), Bio-Rad (Richmond, CA), Becton Dickinson (Cockeysville, MD), and Difco (Detroit, MI).

Plasmid Construction. Plasmids containing the coding sequence of luciferase and the 5′UTR of ODC and AdoMetDC were constructed in the pEUK-C1 expression vector (Clontech, Palo Alto, CA). The plasmid pADC-Luc which contains the 320 base 5′UTR of AdoMetDC was constructed as follows. PCR was carried out on the pSAM320 (15) to create a *Bam*HI site at the 3′ end of the 5′UTR. An antisense primer, 3′-AAGCGATCAGAGTGC-CCCTAGGTTCGACGTGT-5′ (underlining, the *Bam*HI site), and a sense primer corresponding to a sequence in the plasmid vector were used, and the

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[3] The abbreviations used are: ODC, ornithine decarboxylase, AdoMetDC, *S*-adenosylmethionine decarboxylase; DFMO, α-difluoromethylornithine; 5′UTR, 5′ untranslated regions; PCR, polymerase chain reaction; ORF, open reading frame; DMEM, Dulbecco's modified Eagle's medium.

PCR product was cut with *Xho*I and *Bam*HI. The luciferase coding region was obtained from plasmid pGL2 (Promega) by cutting with *Bam*HI and *Sac*I. The pEUK-C1 vector was digested with *Xho*I and *Sac*I. The three pieces were then ligated together to form pADC-Luc. The plasmid pADC(-ATG)-Luc in which the internal ORF in the 5′UTR was destroyed by changing the ATG sequence to CGA was prepared by digesting pSAM320-ATG (15) with *Nde*I, which cuts within the pEUK-C1 vector, and *Nhe*I, which cuts within the 5′UTR of AdoMetDC. A 1983-base pair piece was isolated. pADC-Luc was cut with the same enzymes, and a 5000-base pair piece was isolated and ligated to the 1938-base pair fragment to form pADC(-ATG)-Luc. The plasmid pODC-Luc, which contains 222 bases of the 5′UTR of ODC, was constructed as follows. pEUK-ODC, which contains the ODC 5′UTR and protein coding region in the pEUK-C1 vector,[4] was used as a template for PCR to create a *Bam*HI site at the ODC initiation codon. The same sense primer needed for the construction of pADC-Luc and an antisense primer with the sequence 3′-TTCGTG-TAGCTCTTCCTAGGCGTCGAAATGA-5′ (underlining, the *Bam*HI site) were used. The product of this reaction was digested with *Xho*I and *Bam*HI and combined in a ligation reaction with the pEUK-C1 vector digested with *Xho*I, *Sac*I, and the *Bam*HI/*Sac*I fragment from pGL2 to create the plasmid pODC-Luc. The sequence of all of these plasmids was confirmed by sequencing the 5′UTR regions.

Cell Culture. Cells were grown in DMEM supplemented with 10% fetal calf serum, 4 mM glutamine, and 100 μg/ml penicillin and streptomycin (14). 3T3 pMV7–4E(P2) cells were maintained in 500 μg/ml Geneticin (G418 sulfate; GIBCO/BRL, Gaithersburg, MD). Cell extracts were prepared for enzyme assays, polyamine measurements, or RNA blots as described previously (7).

For plating in soft agar, 5×10^4 cells were resuspended in 2 ml of 0.35% (w/v) agar solution containing DMEM plus 20% fetal calf serum and overlaid onto a 0.5% agar solution in a 35-mm plate. Two days after plating, 2 ml of DMEM supplemented with 20% fetal calf serum was added. Colonies were counted 7 days after plating. When DFMO was added, it was included in the agar when cells were plated and also in the added medium.

Determination of Enzyme Activities and mRNA Levels. ODC and AdoMetDC were assayed by measuring the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine (16) or *S*-adenosyl[*carboxy*-^{14}C]methionine (17), respectively. The mRNA content for these enzymes was determined essentially as described (7) using probes prepared with digoxigenin-labeled UTP and plasmids pCM9 (17) as a template for AdoMetDC and pGEM-ODC (16) as a template for ODC. RNA was detected using a nucleic acid detection kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer's instructions and was quantitated using a laser densitometer.

Luciferase Assays. Cells were transfected 24 h after plating, and transfections were performed for 5 h with 2–5 μg of plasmid/plate using the Lipofectin procedure (GIBCO/BRL) according to the manufacturer's instructions. The cells were harvested 48 h after the start of transfection. Luciferase assays were performed with a luciferase assay system (Promega) and quantitated against a standard curve using purified firefly luciferase (Boehringer Mannheim). Results were expressed as fg luciferase per mg protein. Activities were normalized using a control luciferase plasmid (pGL2; Promega) that was also transfected into each cell line. The reported results are the ratio of luciferase activity obtained with each plasmid divided by the activity obtained with the control plasmid.

Results

ODC activity in 3T3 cells showed a peak at 24 h after plating and then declined over the next 72 h. ODC activity in the P2 cells increased greatly over the first 24 h as the cells attached and began growing and remained at high levels over the entire period measured (Fig. 1). After 72 h in culture, ODC in P2 cells was at least 13-fold higher than that in the parent NIH-3T3 cells (Fig. 1; Table 1). By 96 h, the difference was at least 30-fold. In contrast, there was little difference in the AdoMetDC levels of the two cells lines, which showed a peak at 24 h (Fig. 1; Table 1). The mRNA levels corresponding to either ODC or AdoMetDC were virtually identical in P2 cells and the parent cell line (Table 1). The difference in ODC levels between the P2 and the 3T3 cells was even more striking after longer times in culture. When the cells were grown for 7 days (with the medium changed every 3 days), the level of ODC in the 3T3 cells was 0.005 nmol/mg/30 min and that in the P2 cells was 600 times greater (3.2 nmol/mg/30 min).

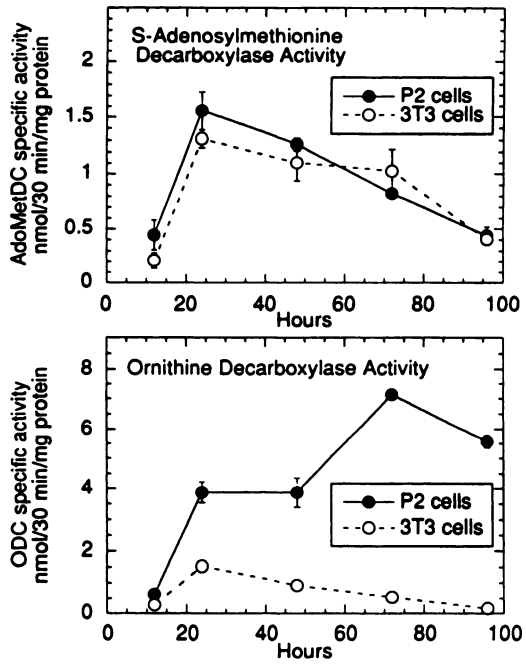

Fig. 1. Activity of ODC and AdoMetDC in 3T3 and P2 cells. Cells were plated at a density of 10^4/cm^2 and grown for various times up to 96 h. At the times indicated, dishes were harvested, and ODC (*bottom*) and AdoMetDC activity (*top*) were measured. *Points*, means of triplicate measurements; *bars*, SD.

Table 1 *Endogenous enzyme activities and RNA levels in NIH 3T3 cells and P2 cells*

Cells were grown and harvested 72 h after plating as described in "Materials and Methods." Enzyme activities are expressed as the mean ± SD of 3 to 7 separate determinations. RNA levels are expressed as absorbance units/μg of total RNA determined using a laser densitometer.

	Enzyme activity (nmol/30 min/mg protein)		RNA levels (units/μg RNA)	
Cell line	AdoMetDC	ODC	AdoMetDC	ODC
NIH 3T3	0.36 ± 0.08	0.25 ± 0.04	1.50	1.59
pMV7-4E(P2)	0.54 ± 0.1	3.37 ± 0.58	1.99	1.75

These results suggest that ODC translation may be increased in the P2 cells as a consequence of the elevated level of eIF-4E. This was examined by measuring the formation of luciferase in these cells after transient transfection of plasmid constructs containing the 5′UTR of ODC or AdoMetDC fused to luciferase (Fig. 2). There was a 2.5-fold increase in luciferase expression in the P2 cells when the 5′UTR came from ODC, but there was no difference in luciferase expression from the constructs containing the AdoMetDC 5′UTR. These results indicate that the 5′UTR from ODC is less inhibitory to translation of the chimeric luciferase mRNA when eIF-4E levels are high and confirm previous suggestions that this initiation factor may be a limiting factor in the translation of mRNAs that have extensive secondary structure. Translation of luciferase mRNAs containing the 5′UTR from AdoMetDC was not increased in the P2 cells. Furthermore, this mRNA was very poorly translated (Fig. 2). A major factor in this very poor translation is the ORF in the 5′UTR (which runs from bases −312 to −297). Removal of this ORF by mutation of the AUG codon improved the expression of luciferase considerably, but there was no

[4] A. E. Pegg and A. Manni, unpublished data.

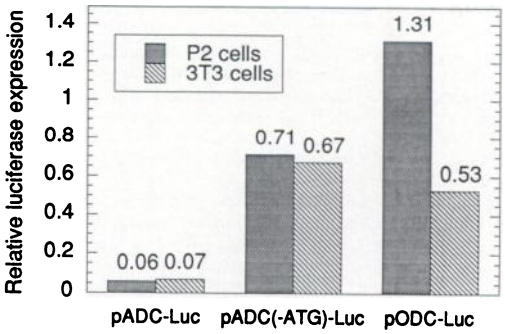

Fig. 2. Expression of luciferase from plasmids containing the 5′UTR from ODC or AdoMetDC transfected into 3T3 or P2 cells. Cells were grown and transfections carried out as described in "Materials and Methods." Results are the means of duplicate assays which agreed within 5% and were normalized by dividing the activities obtained by the activity produced by a control luciferase plasmid. There was no significant difference in the expression of this control plasmid in the 3T3 and P2 cells.

difference between the level of luciferase produced in the two cell lines. Thus, despite the apparent similarity between ODC and AdoMetDC in that they both have long 5′UTRS and are regulated at the level of translation, there is a fundamental difference in this regulation involving the ORF in the case of AdoMetDC and the secondary structure in the case of ODC.

The increased ratio of ODC to AdoMetDC in the P2 cell line was reflected in the polyamine content of the cells and in the culture medium. Compared to the 3T3 cells, putrescine levels in P2 cells were increased 3.5-fold, and spermidine levels increased 2-fold, reaching 5.2 and 18.4 nmol/mg protein, respectively, when measured at 48 h after plating. There was an even larger (7-fold) increase in putrescine in the medium (data not shown).

These results suggest that the high level of ODC expression in the P2 cells may be a critical factor in their transformed phenotype. This was examined by using DFMO to reduce the ODC level and measuring the ability of the cells to form colonies in agar (Fig. 3). DFMO produced a dose-dependent decrease in both ODC activity, measured after 7 days of growth on a monolayer, and in the colony-forming activity of the P2 cells. Colony formation was abolished by 50 μM DFMO. This level of DFMO reduced the ODC activity at 7 days from 3.2 nmol/mg protein to 0.032, although this value was still higher than that in the 3T3 cells at this time.

Discussion

These results provide very strong evidence that ODC translation is regulated by the content of eIF-4E. Not only is the endogenous ODC activity much higher in the P2 cells that overexpress this factor, but the production of luciferase from a mRNA with most of the 5′UTR from ODC was increased in these cells. The level of increased luciferase expression was only 2.5-fold, which is significantly less than the increase in ODC. This may indicate that the coding and 3′UTR sequences of ODC also play a role in its translational efficiency, and there is some previous evidence for this (2). However, it is possible that additional expression of the luciferase-ODC chimera saturates the available eIF-4E in the P2 cells. Based on other studies in which eIF-4E has been implicated in the "melting" of secondary structure in the 5′UTR of other mRNAs, it is likely that this is the mechanism by which increased ODC expression occurs. The lack of increased synthesis of AdoMetDC suggests that the secondary structure of this 5′UTR is not the limiting factor in its translation. This is in agreement with previous work showing that the internal ORF has a powerful influence on AdoMetDC synthesis (5, 15). Even when this ORF is removed, which led to a major increase in luciferase expression from the AdoMetDC-luciferase chimeras, there was still no difference between the response of P2 and 3T3 cells.

The persistently high level of ODC and the increased ODC: AdoMetDC ratio in the P2 cells lead to a much higher content of putrescine in the cells and in the medium. This intracellular overproduction of putrescine may be related to the neoplastic phenotype of these cells. Overexpression of ODC in 3T3 cells has been shown to lead to transformation (10, 11). In these studies, ODC synthesis was driven to very high levels by inserting the complementary DNA into vectors driven by a very strong promoter. A third study, where a lower level of ODC expression which did not by itself cause transformation was obtained, showed that coexpression with c-Ha-*ras* produced a transformed phenotype (18). It is certainly possible that other growth factors/oncogenes may also be increased as a result of the production of high levels of eIF-4E and these may combine with ODC to lead to transformation. However, the fact that reduction of ODC by 50 μM DFMO abolished the anchorage-independent growth of the P2 cells also provides evidence that ODC is important in the neoplastic response. It is unlikely that the well known antiproliferative effect of DFMO (4) is responsible for this effect since the residual ODC activity in the DFMO-treated P2 cells was still greater than that in the parent 3T3 cells, even after exposure to 200 μM DFMO.

There is evidence for increased transcription of the ODC gene in response to some carcinogens, tumor promoters, and oncogenes (1, 8, 19, 20), but the very poor translatability of the ODC gene and the close regulation of this translation by cellular polyamine levels may restrict the expression of ODC activity. Our results show that changes in factors altering translation may be an important factor in ODC expression. Although the overproduction of eIF-4E could lead to the increased synthesis of such oncogenes, our results rule out a second-

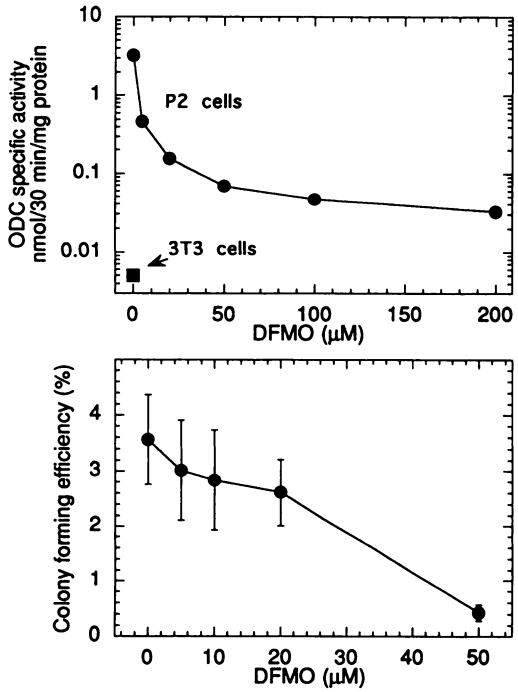

Fig. 3. Effect of DFMO on ODC activity and anchorage-independent colony formation in P2 cells. *Top,* effect of various concentrations of DFMO on ODC activity measured at 7 days after plating. The medium was replaced every 3 days. The values shown are the means of duplicate determinations expressed as the specific activity of ODC (nmol of CO_2 released per 30 min per mg of protein). *Bottom,* effect of DFMO on the ability of the cells to form colonies when grown in soft agar. DFMO was added when the cells were plated and was present throughout the experiment. *Points,* means of from 4 to 6 separate determinations; *bars,* SD. Cloning efficiency in agar was calculated by the number of colonies × 100 divided by the number of cells plated. Colonies are defined as cell aggregates containing more than 10 cells.

ary effect on ODC in P2 cells via its increased transcription. The P2 cells provide an excellent model for studying the effects of polyamine levels on the regulation of translation of ODC by eIF-4E and the possible interaction of this factor with polyamines in influencing the translation of ODC and AdoMetDC mRNA.

It has long been known that polyamines play an essential role in the proliferation of mammalian cells, and the depletion of polyamines inhibits growth of neoplastic cells both *in vitro* and in animal models (1). The additional evidence now available suggesting that high levels of ODC are associated with neoplastic transformation provides strong support for the planned trials of DFMO as a chemopreventive agent in several types of human cancers (reviewed in Ref. 4).

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References

1. Pegg, A. E. Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. Cancer Res., *48:* 759–774, 1988.
2. Grens, A., and Scheffler, I. E. The 5′- and 3′-untranslated regions of ornithine decarboxylase mRNA affect the translational efficiency. J. Biol. Chem., *265:* 11810–11816, 1990.
3. Manzella, J. M., and Blackshear, P. J. Specific protein binding to a conserved region of the ornithine decarboxylase mRNA 5′-untranslated region. J. Biol. Chem., *267:* 7077–7082, 1992.
4. McCann, P. P., and Pegg, A. E. Ornithine decarboxylase as an enzyme target for therapy. Pharmacol. Ther., *54:* 195–215, 1992.
5. Hill, J. R., and Morris, D. R. Cell-specific translation of *S*-adenosylmethionine decarboxylase mRNA. J. Biol. Chem., *267:* 21886–21893, 1992.
6. Lövkvist, E., Stjernborg, L., and Persson, L. Feedback regulation of mammalian ornithine decarboxylase studies using a transient expression system. Eur. J. Biochem., *215:* 753–759, 1993.
7. Shantz, L. M., Holm, I., Jänne, O. A., and Pegg, A. E. Regulation of *S*-adenosylmethionine decarboxylase activity by alterations in the intracellular polyamine content. Biochem. J., *288:* 511–518, 1992.
8. Gilmour, S. K., Robertson, F. M., Megosh, L., O'Connell, S. M., Mitchell, J., and O'Brien, T. G. Induction of ornithine decarboxylase in specific subpopulations of murine epidermal cells following multiple exposures to 12-*O*-tetradecanoylphorbol-13-acetate, merzerein and ethyl phenylpropriolate. Carcinogenesis (Lond.), *13:* 51–56, 1992.
9. Kozak, M. The scanning model for translation: an update. J. Cell Biol., *108:* 229–241, 1989.
10. Auvinen, M., Paasinen, A., Andersson, L. A., and Hölttä, E. Ornithine decarboxylase activity is critical for cell transformation. Nature (Lond.), *360:* 355–358, 1992.
11. Moshier, J. S., Dosescu, J., Skunca, M., and Luk, G. D. Transformation of NIH/3T3 cells by ornithine decarboxylase overexpression. Cancer Res., *53:* 2618–2622, 1993.
12. Manzella, J. M., Rychlik, W., Rhoads, R. E., Hershey, J. W. B., and Blackshear, P. J. Insulin induction of ornithine decarboxylase. Importance of mRNA secondary structure and phosphorylation of eukaryotic initiation factors eIF-4B and eIF-4E. J. Biol. Chem., *266:* 2383–2389, 1991.
13. Lazaris-Karatzas, A., Montine, K. S., and Sonenberg, N. Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5′ cap. Nature (Lond.), *345:* 544–547, 1990.
14. Koromilas, A. E., Lazaris-Karatzas, A., and Sonenberg, N. mRNAs containing extensive secondary structure in their 5′ non-coding region translate efficiently in cells overexpressing initiation factor eIF-4E. EMBO J., *11:* 4153–4158, 1992.
15. Shantz, L. M., and Pegg, A. E. Effect of the 5′ untranslated region on the expression of human *S*-adenosylmethionine decarboxylase (AdoMetDC) and its regulation by inhibitors of polyamine biosynthesis. FASEB J., *7:* A1084, 1993.
16. Lu, L., Stanley, B. A., and Pegg, A. E. Identification of residues in ornithine decarboxylase essential for enzymatic activity and for rapid protein turnover. Biochem. J., *277:* 671–675, 1991.
17. Stanley, B. A., Pegg, A. E., and Holm, I. Site of pyruvate formation and processing of mammalian *S*-adenosylmethionine decarboxylase proenzyme. J. Biol. Chem., *264:* 21073–21079, 1989.
18. Hibshoosh, H., Johnson, M., and Weinstein, I. B. Effects of overexpression of ornithine decarboxylase (ODC) on growth control and oncogene-induced cell transformation. Oncogene, *6:* 739–743, 1991.
19. Bello-Fernandez, C., Packham, G., and Cleveland, J. L. The ornithine decarboxylase gene is a transcriptional target of c-Myc. Proc. Natl. Acad. Sci. USA, *90:* 7804–7808, 1993.
20. Hurta, R. A. R., Greenberg, A. H., and Wright, J. A. Transforming growth factor B_1 selectively regulates ornithine decarboxylase gene expression in malignant H-*ras* transformed fibrosarcoma cell lines. J. Cell. Physiol., *156:* 272–279, 1993.

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