

Acute Increases in Intracellular Putrescine Lead to the Increase in Steady-State Levels of *c-fos*, *c-jun*, *RING3*, and *Id-1* mRNAs

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1. Introduction

For a number of years, researchers have noted that cancer cells have higher levels of ornithine decarboxylase (ODC) activity than do the corresponding noncancerous tissues (1–5). Because increases in ODC activity (and subsequently, the polyamines) occur regularly in the cell cycle and are necessary before successful cell division can occur, the exact role of these increased polyamine levels in tumorigenesis was not completely understood. It was unclear whether the increases in ODC and polyamine levels directly participated in the generation of the cancer phenotype, or whether these increases were only a result of the increased cell cycling that is a hallmark of many cancerous cells and tumors (1–4).

This “chicken or egg” conundrum was clearly resolved by the production of a transgenic mouse that overexpressed ODC only in keratinocytes (5,6). By itself, the overexpression of ODC in these animals was unable to cause the appearance of tumors. When the skin of these transgenic mice was treated with a low dose of the mutagen 7,12-dimethylbenz[a]anthracene, an initiator of *v-Ha-ras*, papillomas were readily formed (7). This observation indicated that elevated ODC and the resulting increases in intracellular polyamines could act as a tumor promoter (taking the place of phorbol esters) in this two-stage model of tumorigenesis (8–10). There have been many other studies (many reviewed in this monograph) that have further implicated ODC overexpression and increased polyamines as a causative factor in other experimental models of cancer development and in the etiology of human cancer.

Although the precise mechanisms by which increases in ODC activity (and intracellular polyamine levels) lead to tumor formation are unknown, it appears likely that there must first be an independent initiating event (mutation) that will allow the elevated polyamines to promote tumor growth. This was also demonstrated when a transgenic mouse that expressed the mutant form of *v-Ha-ras* (that normally results from 7,12-dimethylbenz[a]anthracene exposure) was crossed with the ODC overexpressing

mouse line. Although neither of these two transgenic mouse lines individually bore any tumors, when bred to each other the resulting offspring spontaneously produced papillomas without the need for any further input (11). Additionally, once formed, these tumors could be regressed by the administration of the ODC inhibitor α -difluoromethyl-ornithine (DFMO) in the drinking water of the tumor-bearing mice. When DFMO was removed from the water, the tumors reappeared at the same sites that they had occupied before the DFMO-mediated regression (12). These observations underscore the reversible role that ODC activity and increased polyamine levels play in the promotion and maintenance of these papillomas.

Our hypothesis was that ODC overexpression and sustained elevated polyamine accumulation led to an alteration in net gene expression of a specific subset of genes involved in tumor promotion. Although a number of genes have been shown to be differentially expressed in various tumor tissues, only spermidine/spermine- N^1 -acetyltransferase (SSAT) has been definitively shown to have a polyamine responsive element in its upstream regulatory region (13). Further, in the case of the transgenic mouse model, the overexpression of ODC in the keratinocytes was chronic in nature. These continuously elevated intracellular polyamine concentrations may have, over time, brought about a series of accommodative long-term changes in the metabolism or gene expression patterns in these keratinocytes that were responsible for the tumor promotion.

For these reasons, our laboratory became interested in the subset of genes whose levels of expression are affected in the short term by acute changes (either up or down) in intracellular polyamine pools. In an effort to identify "early" polyamine responsive genes, we developed model systems using the T-Rex system (Invitrogen) to produce a series of human cell lines containing an inducible antizyme-resistant truncated-ODC complementary DNA. The resulting cell lines were named 293 ODC/Tet-Ind (derived from the 293 human embryonic kidney cell line); MCF-7 ODC/Tet-Ind (derived from the MCF-7 human breast cancer cell line); and LNCaP ODC/Tet-Ind (derived from the LNCaP human prostate cancer cell line). In the absence of tetracycline (TET), all of these cell lines had low basal levels of ODC activity and intracellular putrescine. When tetracycline was added to the culture media, ODC activities (and concomitant intracellular putrescine levels) in these cells could be rapidly induced to high levels in a dose-dependent manner (14).

To identify the population of genes whose expression levels are altered by acute increases in intracellular putrescine concentrations, we treated the MCF-7 ODC/Tet-Ind and the 293 ODC/Tet-Ind cells with either TET alone, or in concert with exogenous substrate ornithine (ORN) or inhibitor DFMO. After 12 h (293) or 18 h (MCF-7), the cells were harvested and their polyamine contents determined. Total RNA from these cells were also collected. In both cell lines, the cells receiving TET or TET + ORN had significantly elevated intracellular polyamine levels. In contrast, the cells treated with DFMO and TET had intracellular polyamine levels that were identical to levels measured in control (untreated) cells. The RNA collected from the MCF-7 ODC/Tet-Ind and 293 ODC/Tet-Ind cells were subjected to genome-wide gene expression analysis using Affymetrix U95 human gene chip arrays.

On careful analysis of the resulting data from all treatments and cell types (data not shown), we identified a small number of messenger RNA (mRNA) whose expression

levels were significantly increased in response to elevated intracellular polyamines. Expected and found among these induced genes were *SSAT*, *c-FOS*, and *c-JUN*. However, we also found the dominant-negative transcription factor *ID-1* (inhibitor of differentiation-1) (15–19) and *RING3* (which reportedly interacts with E2F and modulates E2F-mediated transcription) (20,21) to be some of the polyamine-related genes.

In this chapter, we will detail some of the different ways in which polyamine pools can be manipulated to increase the net levels of expression of some of the mRNA, identified via genome-wide expression analysis in another ODC inducible cell line the LNCaP ODC/Tet-Ind cells. Our hypothesis was that the ability of elevated polyamines to increase the net accumulation of specific mRNA should not be cell type-specific.

1.1. A Stably Transfected TET-Inducible ODC System Effectively Increases Intracellular Polyamine Concentrations

To determine the extent of which TET alters intracellular polyamine levels after the induction of ODC in the cultured LNCaP ODC/Tet-Ind cells, the cell culture media was treated for up to 9 h with either 1 $\mu\text{g/mL}$ exogenous TET, 1 $\mu\text{g/mL}$ TET along with 1 mM DFMO, TET, and 1 mM ORN, 1 mM ORN only, or untreated (control). At 3-h intervals, cells were harvested, and their intracellular polyamine concentrations determined.

Figure 1A shows changes in intracellular putrescine. In the untreated cells, the intracellular putrescine levels were essentially unchanged at 6 nmol/mg protein over the 9-h time course. In contrast, the addition of TET caused a marked and steady increase in intracellular putrescine levels for the first 6 h to 160 nmol/mg protein followed by a slight decrease to 100 nmol/mg protein after 9 h. The addition of the ODC inhibitor, DFMO, effectively blocked the TET-mediated accumulation of intracellular putrescine. The addition of 1 mM ORN along with TET produced levels of intracellular putrescine similar to those seen in the TET only cells at 3 and 6 h. However, at 9 h, the TET + ORN cells had continued to accumulate intracellular putrescine concentrations to 250 nmol/mg protein. The addition of substrate ornithine alone in the absence of TET resulted in intracellular putrescine levels that were essentially unchanged from control concentrations.

Figure 1B illustrates the levels of intracellular spermidine over the 9-h time course. In the control, TET + DFMO and ORN-only treated cells exhibited intracellular spermidine levels that remained unchanged. However, in the cells receiving TET or TET + ORN, intracellular spermidine levels continued to decrease throughout the time course. At 9 h, the cells treated with TET + ORN had lost half of their intracellular spermidine, whereas the TET-only cells retained only 5% of the intracellular spermidine relative to the control values. Intracellular spermine values were relatively stable throughout the 9-h time course, with one notable exception being the TET-only cells, which lost 50% of the intracellular spermine between 6 and 9 h.

1.2. Similar Changes in Gene Expression Patterns Seen in Multiple Cell Types After Increases in ODC Activity

Would the genes identified from the genome-wide expression analysis of 293 ODC/Tet-Ind cells and MCF-7 ODC/Tet-Ind cells show altered gene expression in another cell line engineered to overexpress ODC? Using the same experimental treatments

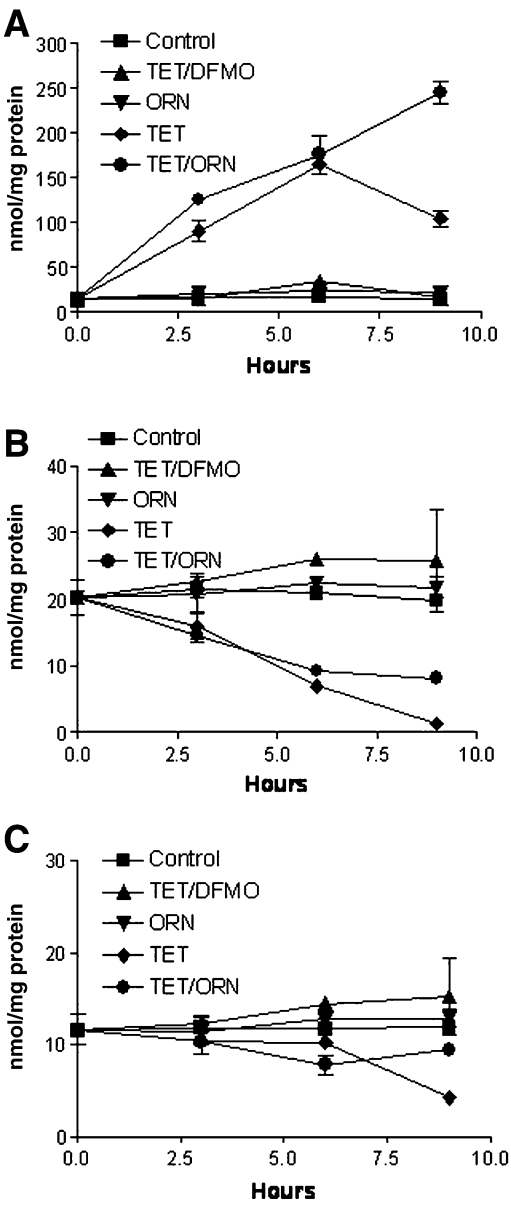


Fig. 1. Intracellular polyamine profile of LNCaP ornithine decarboxylase (ODC)/Tet-Ind cells after ODC induction by tetracycline for 3, 6, and 9 h. The cells were either untreated (control) or given 1 μ g/mL tetracycline (TET), 1 μ g/mL tetracycline and 1 mM α -difluoromethylornithine (DFMO) (TET/DFMO), 1 mM ornithine (ORN), or 1 μ g/mL tetracycline and 1 mM ornithine (TET/ORN). The cell culture media was changed at time zero and contained the treatments indicated. To completely block the activity of ODC, the TET/DFMO cells were preincubated with 1 mM DFMO 1 h before the change of media. Values are means \pm SEM of data repeated in triplicate.

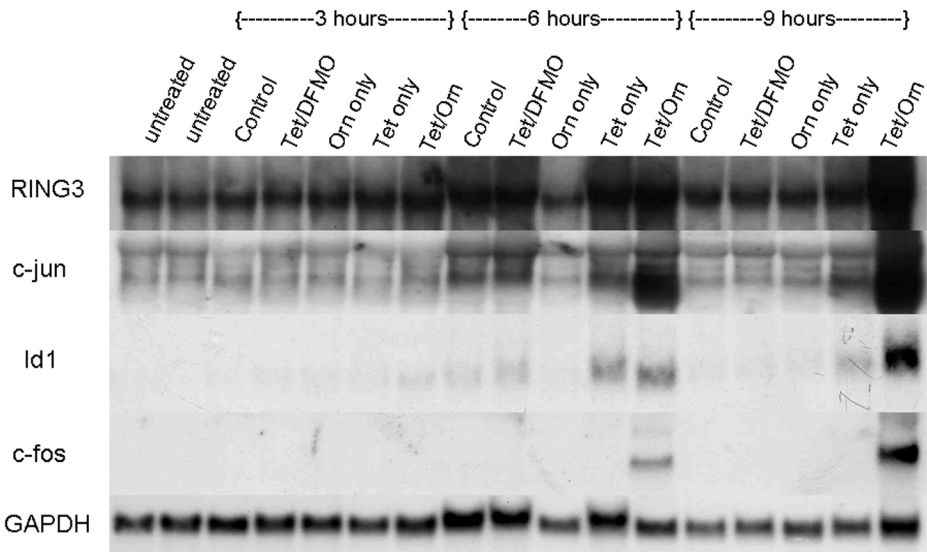


Fig. 2. Northern analysis of LNCaP ornithine decarboxylase (ODC)/Tet-Ind cells after ODC induction by tetracycline (TET) for 3, 6, and 9 h. The cells were either untreated (control) or given 1 $\mu\text{g}/\text{mL}$ TET, 1 $\mu\text{g}/\text{mL}$ tetracycline and 1 mM difluoromethylornithine (DFMO) (TET/DFMO), 1 mM ornithine (ORN), or 1 $\mu\text{g}/\text{mL}$ tetracycline and 1 mM ornithine (TET/ORN). The cell culture media was changed at time zero and contained the treatments indicated. To completely block the activity of ODC, the TET/DFMO cells were preincubated with 1 mM DFMO 1 h before the change of media. Total RNA was isolated and subjected to Northern blot analysis with probes for *RING3*, *c-jun*, *Id-1*, and *c-fos*. Filters were then stripped and reprobed for *GAPDH*.

used previously, the LNCaP ODC/Tet-Ind cells were harvested at 3-h intervals and their steady-state mRNA levels of *RING3*, *c-jun*, *Id-1*, *c-fos*, and *GAPDH* determined. In the Northern blot pictured in Fig. 2, the control cells revealed no change in steady-state mRNA levels during the 9-h time course, whereas the TET-treated cells revealed increased steady-state mRNA levels of *RING3*, *c-jun*, and *Id-1* after 6 h and continued to remain elevated for the duration of the time course. The TET-mediated mRNA elevations of the genes were diminished through the addition of DFMO. The addition of ORN together with TET produced higher levels of steady-state mRNAs in *RING3*, *c-jun*, and *Id-1* genes than in the treatment with TET alone. The ORN + TET-treated cells also produced increased mRNA levels of the *c-fos* gene after 6 and 9 h. The cells treated with ORN alone resulted in no change from the control lanes.

1.3. Exogenous Administration of Putrescine Had No Effect of Cell Viability

To determine whether cell viability was affected by exogenous putrescine, we treated the LNCaP ODC/Tet-Ind cells with increasing exogenous putrescine concentrations for up to 72 h (Fig. 3). Even the cells treated with 30 mM exogenous putrescine had similar doubling times as control untreated LNCaP ODC/Tet-Ind cells for the initial

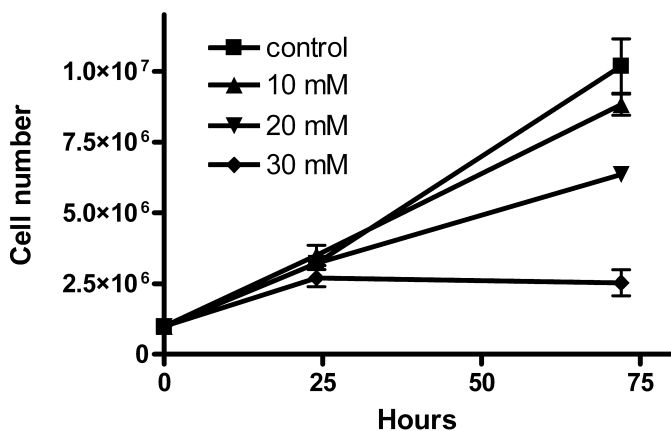


Fig. 3. Growth curve profile of LNCaP ornithine decarboxylase/Tet-Ind cells after the addition of 0, 10, 20, and 30 mM exogenous putrescine for a duration of 72 h. Trypan blue dye was added to a final concentration of 10%, and the viability of the cells was determined by counting blue stained (dead or dying) vs unstained (alive) cells on a hemacytometer. Results are a representative experiment that has been repeated in triplicate.

24 h. A dose-dependent reduction in the growth rate of the cells occurred after 72 h of incubation. However, cell viability remained at 100% after the initial doubling time of 24 h. The TET-mediated cells remained 100% viable throughout the 9-h time course.

1.4. Exogenous Putrescine Administration Capable of Inducing the Same Genes as Increased ODC Activity in a Time- and Dose-Dependent Manner

We wished to determine whether the addition of exogenous putrescine to the cultured media would alter intracellular polyamine concentrations in a similar manner as the TET-mediated ODC overexpression. Figure 4 shows that exogenous putrescine could alter intracellular polyamine concentrations in a dose-dependent manner when cultured LNCaP ODC/Tet-Ind cells were incubated with increasing concentrations of exogenous putrescine for 24 and 36 h. Similar changes in intracellular polyamine concentration occurred during the 24 and 36 h. Intracellular putrescine concentration increased eightfold because of increasing doses of exogenous putrescine (Fig. 4A). In a similar manner to treatments of TET + ORN, intracellular spermidine and spermine concentrations showed a decreasing trend as the exogenous putrescine concentrations increased (Fig. 4B,C). Intracellular spermidine and spermine decreased 90 and 80%, respectively, in cells treated with increasing exogenous putrescine.

Dose-dependent increases in steady-state mRNA levels of *RING3*, *Id-1*, and *c-jun* in response to increasing concentrations of exogenous putrescine after 24 and 36 h are shown in Fig. 5. At the times indicated, the cells were harvested and their total RNA isolated and subjected to Northern blot analysis. Similar changes in the levels of all three of the mRNA species were observed for each of the concentrations of exogenous putrescine after 24 and 36 h of exposure, with the possible exception of the 30 mM

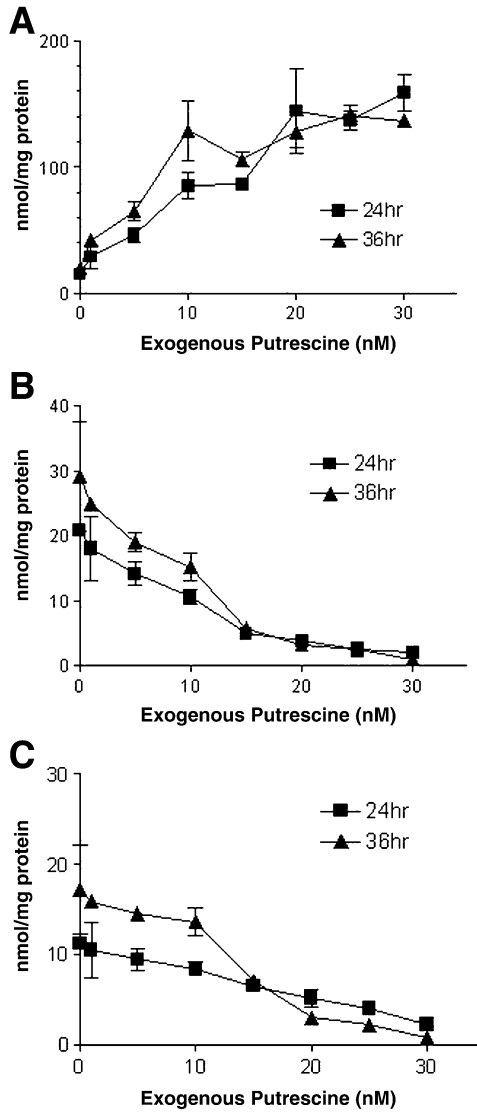


Fig. 4. Intracellular polyamine profile of LNCaP ornithine decarboxylase/Tet-Ind cells after the addition of either 1, 5, 10, 15, 20, 25, or 30 mM exogenous putrescine concentrations for 24 and 36 h. Putrescine was added directly to the media. After 24 or 36 h of incubation in putrescine at the indicated concentrations, the cells were harvested and their intracellular polyamine levels determined. Values are means \pm SEM of data repeated in triplicate.

putrescine-exposed *c-jun*, which had 36-h mRNA levels that were slightly higher than those seen after 24 h. There was no significant increase over control levels in observed mRNA levels in the cells receiving 1 mM exogenous putrescine. However, incubation

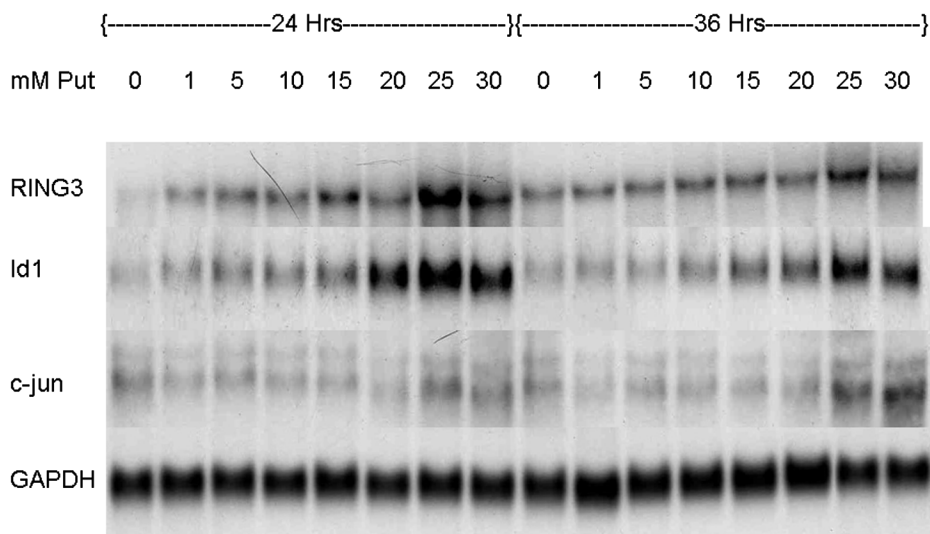


Fig. 5. Northern analysis of LNCaP ornithine decarboxylase/Tet-Ind cells after the addition of putrescine for 24 and 36 h. Putrescine was added directly to the media. After 24 or 36 h of incubation in putrescine at the indicated concentrations, total RNA was isolated and subjected to Northern blot analysis with probes for *RING3*, *c-jun*, *Id-1*, and *c-fos*. Filters were then stripped and reprobed for *GAPDH*.

with 5 mM exogenous putrescine did result in modest increases in the levels of *RING3*, *Id-1*, and *c-jun* mRNA. This indicates that the low end of the induction of these mRNAs was reached when exogenous putrescine levels were 5 mM or higher. This exogenous putrescine concentration resulted in intracellular putrescine levels of 50 nmol/mg protein, intracellular spermidine levels of 18 nmol/mg protein, and intracellular spermine levels of 10 nmol/mg protein (Fig. 4).

To ascertain the time-dependent changes in intracellular polyamine concentrations, the cells were treated with 30 mM exogenous putrescine for 24 h and harvested for polyamine analysis at 3, 6, 9, 12, and 24 h (Fig. 6). The intracellular putrescine concentration increased in a linear fashion for the initial 12 h, reaching a level approx 16-fold higher than measured in the control levels. The putrescine-treated cells also displayed a concomitant loss of spermidine and spermine during the 24 h incubation period. Intracellular spermidine and spermine concentrations decreased 88 and 70% after a delay of 3 and 9 h, respectively. Our cumulative data would indicate that intracellular spermidine and spermine levels do not decrease until intracellular putrescine concentrations reach approx 50 nmol/mg protein.

Cells treated with 30 mM exogenous putrescine were harvested for Northern blot analysis at 3, 6, 9, 12, and 24 h (Fig. 7). After 3 h of treatment, *Id-1* steady-state mRNA levels increased twofold from the control levels. The *Id-1* mRNA levels continued to increase throughout the 24-h time course, reaching a level of 15-fold higher than in control cells. *RING3* and *c-jun* mRNA levels also increased during the time course,

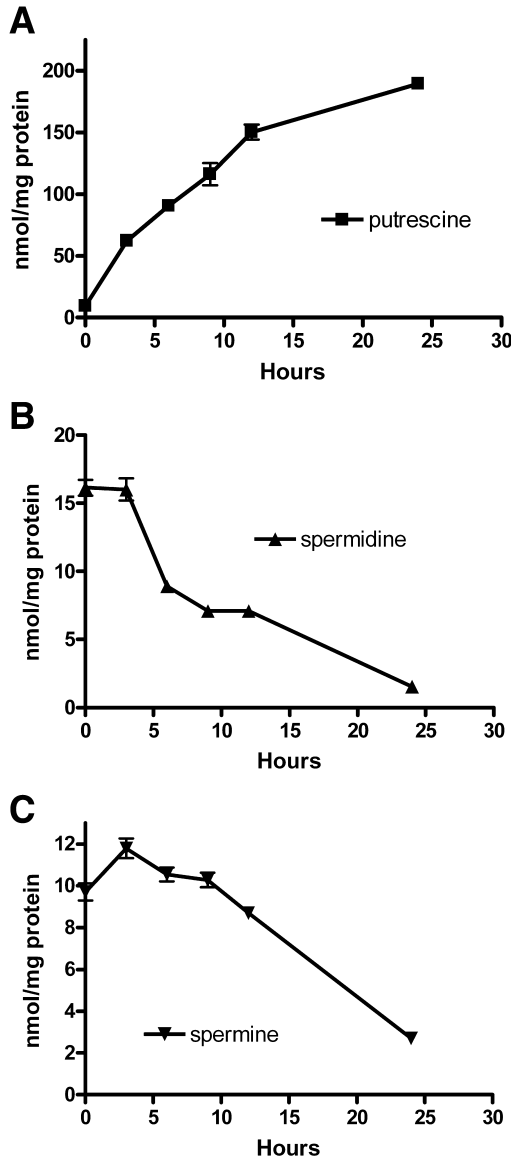


Fig. 6. Intracellular polyamine profile of LNCaP ornithine decarboxylase/Tet-Ind cells after the addition of 30 mM exogenous putrescine for 3, 6, 9, 12, and 24 h. Putrescine was added directly to the media. After incubation in putrescine for the times indicated, the cells were harvested and their intracellular polyamine levels determined. Values are means \pm SEM of data repeated in triplicate.

although not to the same degree as observed with *Id-1*. In contrast, *c-fos* (unlike with TET-mediated ODC overexpression treatments) yielded no consistent change of mRNA levels with exogenous putrescine treatments. These results showed that the *Id-1*,

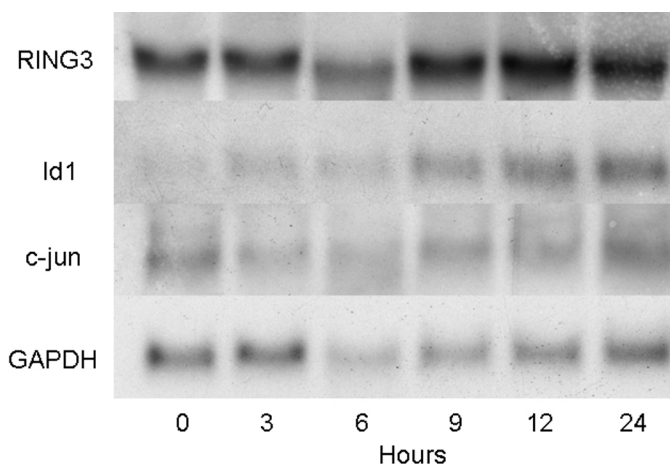


Fig. 7. Northern analysis of LNCaP ornithine decarboxylase/Tet-Ind cells after the addition of 30 mM exogenous putrescine for 3, 6, 9, 12, and 24 h. Putrescine was added directly to the media. After incubation in putrescine for times indicated, total RNA was isolated and subjected to Northern blot analysis with probes for *RING3*, *c-jun*, *Id-1*, and *c-fos*. Filters were then stripped and reprobed for *GAPDH*.

RING3, and *c-jun* genes increased in a time- and dose-dependent manner with exogenous putrescine. *Id-1* showed the most dramatic increases in steady-state mRNA levels, while *RING3* and *c-jun* were less affected. The *c-fos* mRNA levels were not visible after treatment with exogenous putrescine, but again ODC overexpression did lead to increases in *c-fos* (Fig. 2).

1.5. Increased SSAT Activity is Not Responsible for Induction of These Genes

The observed reduction in intracellular spermidine and spermine after treatment with exogenous putrescine or TET-mediated ODC overexpression might have been a result of increased SSAT activity. To determine the extent to which putrescine induces SSAT activity, the LNCaP ODC/Tet-Ind cells were grown for 24 h in media containing either 15 mM exogenous putrescine or 10 μ M DENSPM (N^1 , N^{11} -diethylnorspermine, a spermine analog that induces high levels of SSAT activity) (30) (Table 1) before being harvested and assayed for SSAT activity. The cells receiving exogenous putrescine displayed an eightfold higher SSAT activity than the control cells (Table 1). The DENSPM-treated cells showed an 18-fold higher SSAT activity than the control cells. Our previous studies have shown that TET-mediated ODC overexpressing cells also raised SSAT activity 115-fold higher than control treated cells (data not shown).

ODC overexpression and exogenous putrescine raised intracellular putrescine levels, increased SSAT activity, and further caused a reduction in intracellular spermidine and spermine. We wished to determine whether it was the increase in intracellular putrescine or the reduction in intracellular spermidine or spermine that led to the increase in *RING3*, *Id-1*, and *c-jun* mRNA levels. Because DENSPM caused an increase in SSAT coupled with a reduction of intracellular putrescine (as well as spermidine and spermine), yet did

Table 1

SSAT Activity, Intracellular Polyamine Profile, and Relative Expression Levels of the Genes in LNCaP ODC/Tet-Ind Cells After Treatment of 15 mM Exogenous Putrescine or 10 μ M DENS PM for 24 h

	Control	Exogenous putrescine	DENS PM
SSAT activity (pmol [14 C]-acetylspermidine formed/mg protein \times hour)	162.6 \pm 3.2	1301.5 \pm 142.7	2718.2 \pm 218.6
Putrescine (nmol/mg protein)	15.2 \pm 0.8	122.1 \pm 8.5	11.9 \pm 0.9
Spermidine (nmol/mg protein)	23.1 \pm 0.9	7.7 \pm 0.9	2.1 \pm 0.1
Spermine (nmol/mg protein)	14.7 \pm 0.4	9.9 \pm 1.1	4.9 \pm 0.4
<i>RING3</i> (relative expression)	1	3.5	1.1
<i>Id-1</i> (relative expression)	1	5.8	0.8
<i>c-jun</i> (relative expression)	1	2.2	1
<i>c-fos</i> (relative expression)	1	0.9	0.9

not alter the specific mRNA levels (Table 1), we concluded that increased intracellular putrescine was mediating the alteration of the mRNA levels.

To study the early changes in patterns of gene expression after acute increases in intracellular putrescine concentration, we performed Affymetrix gene chip analysis on mRNAs from human 293 and MCF-7 cell lines containing a stably transfected TET-inducible ODC construct. Among the genes identified as upregulated after induction of ODC in the presence of substrate ORN were *c-fos*, *c-jun*, *RING3*, and *Id-1*. Using another human cell line (LNCaP) containing this inducible ODC construct to confirm the previous observations, we analyzed the changes in the patterns of expression of these genes after alterations in intracellular polyamine pools mediated either by over-expression of ODC or administration of exogenous putrescine. Large increases in intracellular putrescine, in addition to inducing these genes, were accompanied by decreases in intracellular spermidine and spermine. Studies with the inducer of SSAT, DENS PM, mirrored the decreases in intracellular spermidine and spermine without the increase in intracellular putrescine or induction of the genes. Our data indicate that the threshold of induction for these mRNAs is time- and dose-dependent and that, in the short term, intracellular putrescine levels of at least 50 nmol/mg proteins for a period longer than 3 h were capable of significantly increasing the steady-state levels of the mRNAs coding for these growth or cancer-related genes.

2. Possible Mechanisms and Conclusion

Several mechanisms of polyamine-mediated induction of gene expression have been proposed. Some studies have suggested a mechanism involving changes in DNA structure and conformation either through histone acetylation or by enhancing the interaction between nuclear transcription factors and steroid hormone receptors (22–25).

A polyamine-responsive element (PRE) upstream of the transcription start site of SSAT has been identified as another mechanism of how polyamines may modulate

transcription (13). NF-E2-related transcription factor 2 is a nuclear factor that has been reported to bind to the PRE of the *SSAT* gene. Two other proposed ligands of the PRE are the mammalian homolog of the *Arabidopsis* COP 9 signalosome subunit 7a and the polyamine modulated factor 1 (26–28). The exact mechanisms by which these ligands modulate the transcription of *SSAT* have yet to be determined.

Data from other investigators demonstrating changes in specific gene expression after polyamine depletion with DFMO suggest that intracellular polyamines regulate the expression of specific genes (29–37). To lower polyamine levels, the most often used approach was to use DFMO to deplete intracellular putrescine, which would then result in decreased intracellular polyamine pools, mRNA, and the resulting protein product of the polyamine-responsive genes. Additionally, this DFMO effect was ablated by the simultaneous administration of exogenous polyamines (30–37,38). This depletion model has revealed decreases in steady-state expression levels of the RNA coding for *c-myc*, *c-fos*, *c-jun*, *4E-BP1*, *SSAT*, and *IκBα* in a number of different cell lines (30–37,39).

Our data show that increases in intracellular putrescine lead to net changes in levels of gene expression. Two distinct methods were used to raise intracellular putrescine concentrations in LNCaP ODC/Tet-Ind cells and increase the steady-state levels of *RING3*, *Id-1*, *c-jun*, and *c-fos* mRNAs. Either TET-mediated ODC overexpression or the administration of exogenous putrescine were capable of producing sustained increases in intracellular putrescine and elevated steady-state mRNA levels. Although we have yet to identify the mechanisms by which these mRNAs are induced by polyamines, we are in the process of analyzing the upstream regions of these genes for elements capable of activating reporter gene transcription under conditions of elevated intracellular polyamine levels.

It is interesting to note that the genes identified as upregulated by our microarray analysis of cells with elevated intracellular putrescine had been previously shown to be involved either in growth or cancer. AP-1 factors *c-jun* and *c-fos* have been well established in their involvement in tumorigenesis (40). In addition to transcriptional and translational regulation appearing to stem from the *ras* signaling pathway (40), other investigators have demonstrated that exogenous polyamines are capable of causing increases in *c-fos* and *c-myc* gene expression (37).

RING3 (really interesting new gene 3) (recently renamed BRD2), is a serine-threonine kinase that participates in nuclear protein complexes associated with E2F (20). *RING3* has been shown to transactivate E2F-dependent cell-cycle regulatory genes. In concert with activated *ras*, *RING3* was also shown to induce the transformation of NIH/3T3 cells. It has also been speculated that activated *ras* may mediate the phosphorylation of inactive *RING3* (21).

Id-1 (inhibition of differentiation) belongs to the Id family of proteins, which are known to bind with basic helix–loop–helix transcription factors and prevent them from binding DNA. The net effect of *Id-1* binding is a dominant negative regulation of the bound transcription factor and the silencing of the associated genes. These target genes are generally associated with lineage-specific expression and differentiation (41). *Id-1* gene expression has been shown to be elevated in a number of primary tumors vs

normal control tissue specimens (40). In a review by Norton and colleagues (41), *Id-1* regulation was found to be regulated by the expression of immediate early gene *EGR-1*; however, *Id-1* protein is involved in a negative feedback loop that downregulates the formation of *ECR-1* and *c-fos*. This may explain the decrease in expression of *c-fos* mRNA we observed at later time points when the expression of *Id-1* was highest.

At first, we were unsure whether the changes in gene expression observed were the result of the increased intracellular putrescine stemming from the TET-mediated overexpression of ODC or the concomitant decreases in the other polyamines. The data in Table 1 strongly suggest that the decreases in intracellular spermine and spermidine observed after ODC induction did not play a substantial role in the inductions of *RING3*, *Id-1*, *c-jun*, or *c-fos*.

Our data indicate that only when intracellular putrescine levels were significantly increased could elevated steady-state mRNA levels of *RING3*, *Id-1*, *c-jun*, and *c-fos* be measured. The induction of these mRNAs appears to occur when intracellular putrescine concentrations greater than 50 nmol/mg protein are sustained for three or more hours. A further refinement of the putrescine levels and times of exposure that are necessary for the induction of these genes is under way in our laboratory.

Acknowledgments

We would like to thank Dr. Susan K. Gilmour from the Lankenau Institute for Medical Research for her helpful insights. DENSPM was graciously given as a gift from Dr. Carl W. Porter from the Roswell Park Cancer Institute. This work was supported by NIH grant CA79909 to Craig V. Byus.

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Polyamine Cell Signaling

Physiology, Pharmacology, and Cancer Research

Wang, J.-Y. (Ed.)

2006, XIV, 490 p., Hardcover

ISBN: 978-1-58829-625-2

A product of Humana Press