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ORNITHINE DECARBOXYLASE OVER-EXPRESSION STIMULATES MITOGEN-ACTIVATED PROTEIN KINASE AND ANCHORAGE-INDEPENDENT GROWTH OF HUMAN BREAST EPITHELIAL CELLS

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In these experiments we tested the hypothesis that constitutive activation of polyamine(PA) biosynthesis may contribute to mammary carcinogenesis. Spontaneously immortalized normal human MCF-10A breast epithelial cells were infected with the retroviral vector pLOSN containing a cDNA which codes for a truncated and more stable ornithine decarboxylase (ODC), the rate-limiting enzyme in PA synthesis. Upon chronic selective pressure with α-difluoromethylornithine (DFMO) (an irreversible inhibitor of ODC), infected MCF-10A cells exhibited an approximately 250-fold increase in ODC activity, which persisted despite discontinuation of DFMO. ODC-over-expressing MCF-10A cells showed a modest decrease in S-adenosylmethionine decarboxylase and an increase in spermidine/spermineN^1-acetyltransferase. Analysis of cellular PA profile revealed a selective accumulation of putrescine without alterations in spermidine and spermine contents. Lesser degrees of increased ODC activity were obtained reproducibly by re-exposing the cells to incremental small doses of DFMO. We observed a bell-shaped dose-related positive effect of ODC activity on clonogenicity in soft agar of MCF-10A cells. Since anchorage-dependent growth was actually reduced, such positive influence on this feature of transformation was not a non-specific consequence of a growth advantage provided by ODC over-expression. In addition, we observed a close parallelism between the dose-dependent effects of ODC expression on clonogenicity and activity of the ERK-2 kinase, a central element of the MAPK cascade. Our data demonstrate an interaction between PA and the MAPK signalling pathway and suggest that the latter may be involved in ODC-induced transformation of mammary epithelial cells. ***Int. J. Cancer, 70:175–182, 1997.***
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There is evidence to indicate that polyamines (putrescine, spermidine and spermine) are critically involved in breast-cancer-cell proliferation (Manni and Wright, 1984). Furthermore, we have shown that activation of polyamine biosynthesis may lead to tumor progression characterized by the acquisition of a less hormone-responsive and more aggressive breast cancer phenotype (Manni *et al.*, 1995*a, b, c*). On the basis of these findings and the increasingly recognized oncogenic role of ODC (Clifford *et al.*, 1995), the first rate-limiting enzyme in polyamine biosynthesis, we hypothesized that activation of the polyamine pathway may contribute to breast cancer development. To test this hypothesis, we induced ODC over-expression in the spontaneously immortalized MCF-10A human mammary epithelial cell line. MCF-10A cells, which exhibit a completely normal phenotype (Soule *et al.*, 1990), have been extensively used to test the influence of activation of specific genes on the process of mammary carcinogenesis (Basolo *et al.*, 1991). The potential clinical relevance of this experimental system is highlighted by the resemblance of its progressive stages of transformation to those of the human disease, *i.e.*, hyperplasia without and with atypia, carcinoma *in situ*, and infiltrative cancer (Miller *et al.*, 1993).

Our results indicate that induction of ODC over-expression in MCF-10A cells confers anchorage-independent growth and that this effect may be mediated through the MAPK signalling cascade.

MATERIAL AND METHODS

Cell lines and culture conditions

The parent MCF-10A1 cell line (Karmanos Cancer Institute, Detroit, MI) and its genetically engineered derivatives, were grown in Dulbecco's minimal essential medium (DMEM)/F-12 (1:1) supplemented with 5% equine serum, 0.1 µg/ml cholera toxin, 10 µg/ml insulin, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B (GIBCO, Grand Island, NY), 0.5 µg/ml hydrocortisone (Sigma, St. Louis, MO) and 10 ng/ml epidermal growth factor (Collaborative Research, Palo Alto, CA).

Infection technique and DFMO selection

MCF-10A1 cells were infected either with the control retroviral expression vector pLXSN or with the same vector in which a 1.8-kb mouse ODC cDNA had been sub-cloned into the EcoRI restriction site (Clifford *et al.*, 1995). This cDNA codes for a more stable enzymatically active truncated ODC molecule (Ghoda *et al.*, 1989). We empirically observed that, in order to maintain ODC over-expression, it was necessary to chronically expose our infected cells to DFMO treatment. Therefore, ODC-infected MCF-10A1 cells were grown in the presence of 0.1 mM DFMO. The drug was washed off for 3 days or longer before the cells were plated in the experimental dishes. We have reported that a similar experimental approach was necessary to preserve ODC over-expression in transfected MCF-7 breast-cancer cells (Manni *et al.*, 1995*c*).

Anchorage-dependent growth

In these experiments, the cells were plated in triplicate at a density of 4×10^4 cells per 35-mm dish in the various experimental conditions described in ''Results'' and in the figure legends. Triplicate dishes were harvested 24, 48, 72 and 96 hr after plating. The number of cells was counted using a Coulter (Hialeah, FL) counter.

Anchorage-independent growth

Clonogenicity in soft agar was tested as described (Manni *et al.*, 1995*a*). Cells were plated in triplicate at a density of 1.5×10^4 cells per 35-mm dish. The number of colonies (aggregates greater than 50 cells) was scored after 21 to 25 days.

Measurement of cellular enzymatic activity and polyamine levels

In these experiments, the cells were plated in duplicate at a density of 3×10^5 cells per 100-mm dish. For determination of enzymatic activity, the cells were washed 3 times with ice-cold PBS and then re-suspended in buffer containing 50 mM Tris, 2.5 mM DTT, 0.1 mM EDTA, pH 7.5, and stored at −70°C until use. At the time of the assays, the cells were frozen and thawed twice. The cell lysates were centrifuged at 13,600 *g* for 20 minutes.

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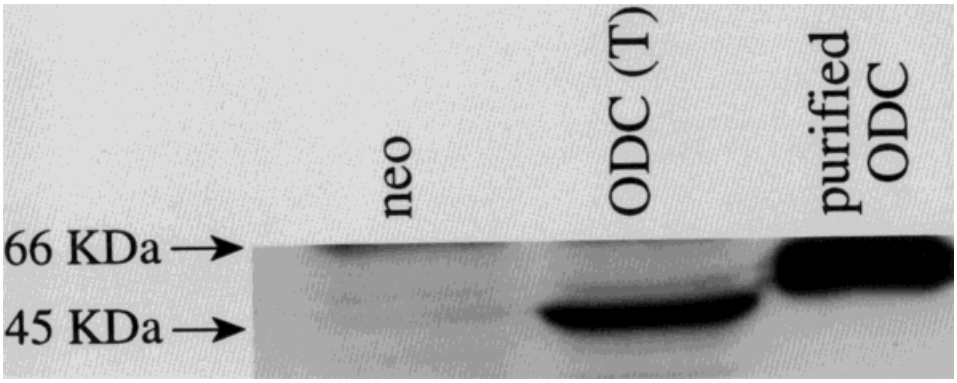

FIGURE 1 – Western-blot analysis of extracts of MCF-10A cells infected either with the control vector (neo) or with the vector containing the truncated ODC cDNA (ODC[T]). Western analysis was performed as reported (Clifford *et al.*, 1995) using an anti-ODC anti-serum provided by Dr. A.E. Pegg (Hershey Medical Center). Note the expected lower MW (47 kDa *vs.* 51 kDa) of the truncated exogenous ODC compared with the recombinant native enzyme (100 ng) run in parallel. The ODC anti-serum was not sufficiently sensitive to detect the much lower level of endogenous ODC expressed by the neo-infected cells.

TABLE I – LEVELS OF POLYAMINE METABOLIC ENZYMES IN MCF-10A MAMMARY CELLS INFECTED EITHER WITH THE CONTROL VECTOR pLXSN (NEO) OR WITH THE SAME VECTOR CONTAINING THE ODC cDNA (ODC)[1]

Groups	ODC (nmol/mg)	SAMDC (nmol/mg)	SSAT (pmol/mg)
neo	1.6 ± 0.2	1.0 ± 0.06	49.8 ± 5.1
ODC	378 ± 31*	0.79 ± 0.03*	75.4 ± 4.1*

[1]ODC-infected MCF-10A cells were chronically (≥2 months) treated with DFMO (0.1 mM). The drug was washed off 3 days before plating the cells in the experimental dishes. Cultures were harvested 48 hr later at 50 to 60% confluence. Data represent means ± SD of 3 replicate experiments, each conducted in duplicate. Enzymatic activities of wild-type MCF-10A cells were comparable with those of neo-infected cells (data not shown).

*$p < 0.0001$ *vs.* control neo cells.

Activity of ornithine decarboxylase, SAMDC and SSAT was determined in the cytosolic fraction according to standard techniques in routine use in our laboratory (Manni *et al.*, 1995*a, b, c*). For measurement of polyamines, the cells were re-suspended in 0.2 N perchloric acid and kept at 4°C overnight. The suspension was then centrifuged at 800 *g* for 15 min and the supernatant stored until the time of the assay. Polyamines were determined by high-pressure liquid chromatography with fluorometry as described (Manni *et al.*, 1995*a, c*).

Western analysis

Western blot analysis of the cell extracts was performed as recently described by us (Manni *et al.*, 1995*c*) using an ODC anti-serum kindly provided by Dr. A.E. Pegg (M.S. Hershey Medical Center, Hershey, PA).

Measurement of ERK2 kinase activity

Exponentially growing cells were harvested by rinsing twice with cold PBS and re-suspension in cold lysis buffer (1% Triton X-100, 10 mM Tris, pH 7.6, 50 mM NaCl, 30 mM Na pyrophosphate, 50 mM NaF, 1 mM phenylmethyl-sulfonyl fluoride, 1 mM Na orthovanadate, 0.1% aprotinin, 5 mM benzamidine, 1 mM EGTA, 100 nM okadaic acid). Cell suspensions were transferred to microcentrifuge tubes, sonicated on ice for 10 sec, then centrifuged for 30 min at 14,000 *g*, 4°C. Lysates containing 500 µg of protein were immunoprecipitated with anti-ERK2 anti-serum (Santa Cruz Biotechnology, Santa Cruz, CA) and the immune complexes were captured with protein-A agarose (Santa Cruz Biotechnology). Immunoprecipitates were washed once with lysis buffer and twice with TBS wash buffer (TBS + 1 mM Na orthovanadate, 5 mM benzamidine). Kinase activity was assayed by incubating the beads with 30 µl of reaction buffer (30 mM HEPES, 10 mM Mg Cl_2, 1 mM DTT, 20 µm ATP, 0.2 µCi/µl ^{32}P-ATP, 5 mM benzamidine, 0.2 mg/ml myelin basic protein (MBP) (Upstate Biotechnology, Placid, NY). After 30 min at 30°C, the reaction was terminated by the addition of 30 µl Laemmli sample buffer (Laemmli, 1970). The phosphorylated proteins were resolved by 15% SDS-PAGE, transferred electrophoretically to a 0.45-µm nitrocellulose membrane and visualized by autoradiography (Kodak XARs film).

Statistical methods

Levels of enzymatic activity in ODC-over-expressing and control cells were compared by *t*-tests. Comparison of ODC activity was performed after logarithmic transformation.

Analysis of variance (ANOVA) was used to analyze cell-count data from the anchorage-dependent growth experiments. All analyses of cell-count data were performed after logarithmic transformation.

RESULTS

ODC, SAMDC, SSAT activity and polyamine profiles of control and infected MCF-10A cells

In the absence of DFMO selection, ODC over-expression in our infected cells was modest (approx. 2–5-fold) and only transient (approx. 1–2 weeks). Chronic exposure of ODC-infected cells to DFMO, however, resulted in a marked increase in ODC activity (approx. 250-fold) (Table I). This degree of over-expression persisted for approximately one month after removal of DFMO before ODC activity started to decline and returned close to control levels by the end of 2 months (data not shown). Table I also shows that, as a result of ODC over-expression, there was a modest but significant decrease in SAMDC (approx. 20%), while the level of SSAT activity increased by approximately 35%. Since chronic exposure to DFMO has been shown to induce over-expression of endogenous ODC, primarily through DNA amplification (Leinonen *et al.*, 1987), we considered it important to determine whether the high enzymatic activity observed in our cells was due to expression of the infected or the endogenous ODC. We took advantage of the lower molecular weight of the truncated ODC to address this issue by Western analysis. The results indicate that it was, indeed, the infected gene that was over-expressed in our cells (Fig. 1). Interestingly, we obtained similar results in MCF-7 breast-cancer cells induced to over-express ODC by using a combined transfection and DFMO-selection pressure approach (Manni *et al.*, 1995*c*). Figure 2 depicts the cellular polyamine levels of control and ODC-infected MCF-10A cells. ODC over-expression resulted in selective accumulation of putrescine, without modification of spermidine and spermine content. The lack of alteration in the

cellular levels of the 2 distal polyamines probably results from the compensatory changes in SAMDC and SSAT activity described above.

Expression of very high levels of ODC activity is known to be associated with cell toxicity (Tome *et al.,* 1994). It is therefore important to carefully analyze the dose-response relationship between level of ODC expression and the resulting cellular phenotype. To address this issue, we attempted to control the degree of ODC activity in our MCF-10A cells by treating the maximally over-expressing cells with increasing doses of DFMO (Fig. 3). The fold increase in enzyme activity compared to neo-infected cells was 267, 212, 31, 10, 7, and 4 in the absence and in the presence of the indicated increasing doses of DFMO.

Growth properties in liquid culture of control and ODC-over-expressing MCF-10A cells

Figure 4*a* shows anchorage-dependent growth of control and ODC-over-expressing MCF-10A cells in the presence and in the absence of serum. As can be seen, cell proliferation was significantly reduced ($p < .0001$) by ODC over-expression in both experimental conditions. Next, we assessed anchorage-dependent growth in the presence and in the absence of EGF. The rationale of this experiment was dictated by the observation that reduced EGF requirement for growth is a feature of MCF-10A cells partially transformed by oncogenic *ras* (Basolo *et al.,* 1991). We wanted to test whether a similar phenotype resulted from ODC over-expression. The results indicate that sensitivity to EGF administration was similar in neo- and in ODC-infected MCF-10A cells (Figs. 4*b,* 5). Again, ODC over-expressing cells grew significantly less ($p < 0.0001$) in the presence and in the absence of EGF (Figs. 4*b,* 5). Figure 5 also shows the influence of variable degrees of increased ODC activity on anchorage-dependent growth. The data indicate that reduced growth occurred at all levels of ODC over-expression without evidence of a dose-response relationship. The levels of cellular ODC activity measured in the experimental conditions depicted in Figure 5 are summarized in Table II. The data documented the variable degree of ODC overexpression exhibited by our cells and, in addition, show a clear positive effect of EGF on the levels of enzymatic activity.

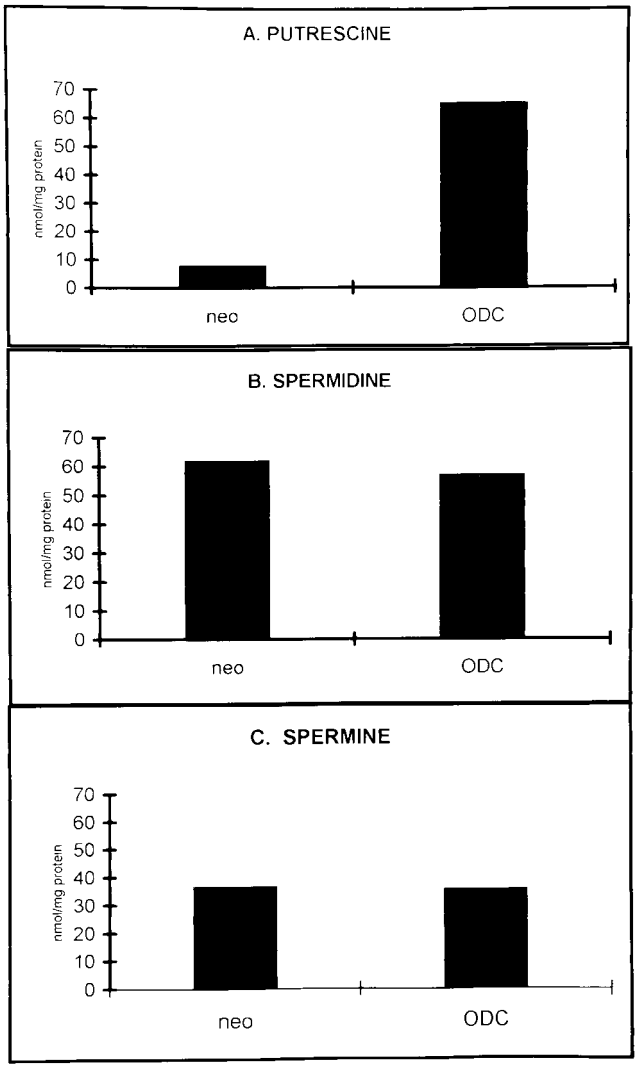

FIGURE 2 – Polyamine levels in MCF-10A cells infected with either pLXSN (neo) or pLOSN (ODC) and maintained in the continuous presence of DFMO (0.1 mM, ≥2 months). Before plating in the experimental dishes, DFMO was washed off for 3 days. Bars represent the mean of duplicate measurements. Similar results were obtained in 2 additional replicate experiments.

Clonogenicity in soft agar of control and ODC-over-expressing MCF-10A cells

Next, we assessed anchorage-independent growth as an indicator of the transformed phenotype conferred by ODC over-expression. As expected, control pLXSN-infected MCF-10A cells exhibited minimal clonogenicity. In contrast, ODC-over-expressing cells (approx. 12-fold over control) acquired the ability to form colonies in soft agar (Fig. 6). To analyze the dose-response relationship between ODC over-expression and clonogenicity, we plated in soft agar pLOSN-infected MCF-10A cells whose degree of ODC activity was titrated by increasing doses of DFMO (Fig. 7). As can be seen, maximal clonogenicity comparable to that induced by the mutated *ras* (neoT), was observed with moderate degrees of ODC over-expression (6–11-fold). Interestingly, in the presence of marked ODC over-expression (250-fold) clonogenicity in soft agar was lost.

Activity of the MAPK cascade in control and ODC-over-expressing MCF-10A cells

In view of the increasingly recognized role of polyamines in multiple cellular phosphorylative events (Hölttä *et al.,* 1994), we were interested in determining whether ODC over-expression would influence the activity of the MAPK signalling cascade. Figure 8 shows that the kinase activity of ERK-2, a key element of this pathway, is greatly enhanced (approx. 6-fold) by moderate (6–11-fold) degrees of ODC over-expression associated with induction of clonogenicity in soft agar (Fig. 7). It should be noted that a more modest increase in ERK-2 activity (approx. 2-fold) was also observed in MCF-10A cells maximally over-expressing ODC (250-fold, Fig. 8). A similar activation of ERK-2 kinase was also exhibited by *ras*-transformed cells (neoT) (Fig. 8), which, in contrast to cells over-expressing ODC 250-fold, were able to form colonies in soft agar (Fig. 7). This finding suggests that additional mechanisms other than modest stimulation in ERK-2 kinase are responsible for *ras*-induced transformation.

DISCUSSION

Growing evidence indicates that ODC and polyamines are intimately involved in carcinogenesis. ODC activity, which is transiently increased upon growth-factor exposure, becomes constitutively activated during cell transformation by chemical carcinogens (Gilmour *et al.,* 1987), viruses (Haddox *et al.,* 1980) and oncogenes (Sistonen *et al.,* 1989). This increase in ODC activity probably represents a crucial step in the transformation process rather than an epiphenomenon, since oncogene-induced transformation can be blocked by an ODC anti-sense strategy (Auvinen *et al.,* 1992) or by a specific enzyme inhibitor (Hölttä *et al.,* 1993). More directly, forced constitutive expression of ODC has been shown to lead to transformation of fibroblasts (Moshier *et al.,* 1993) and,

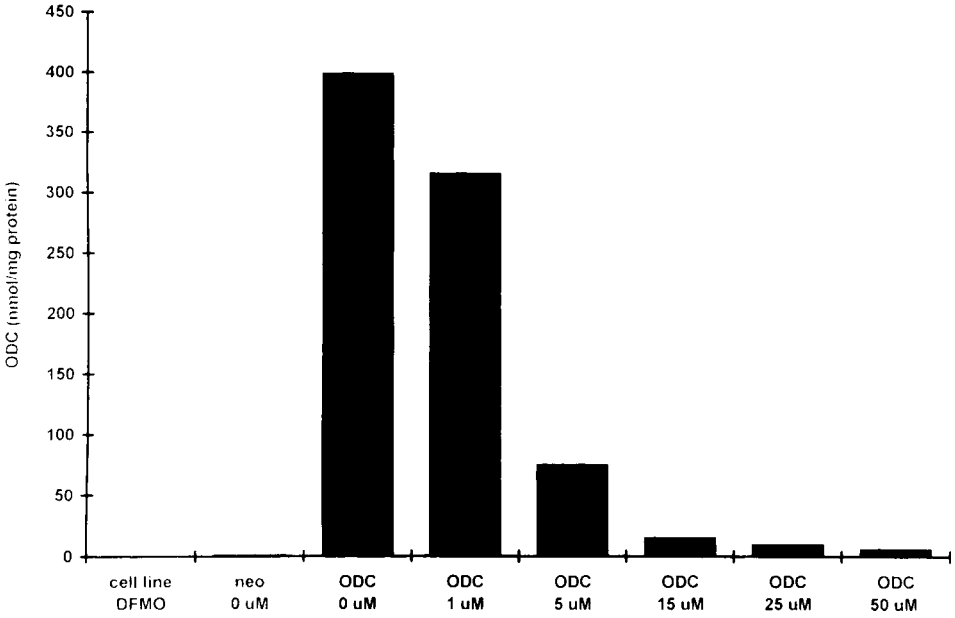

FIGURE 3 – Modulation of ODC activity of MCF-10A cells with graded concentrations of DFMO. MCF-10A cells infected with pLOSN (ODC) and maintained chronically with DFMO (0.1 mM) were washed off DFMO for 3 days. They were subsequently plated in the absence or in the presence of the indicated concentrations of DFMO. The cells were harvested 48 hr later for ODC activity measurement. Bars represent means of triplicate dishes. Similar results were obtained in additional replicate experiments.

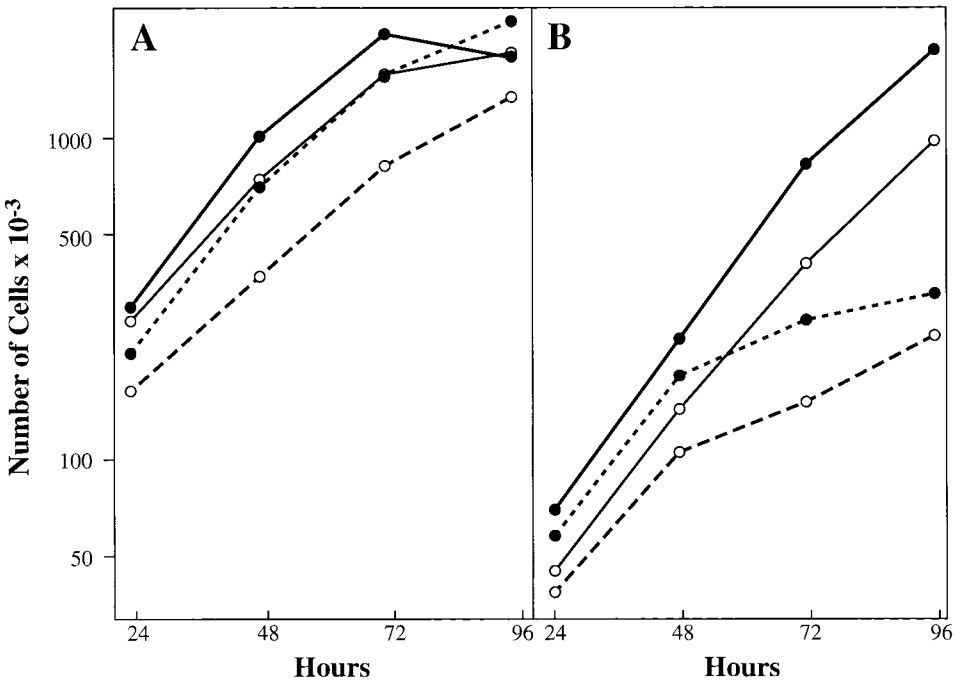

FIGURE 4 – Anchorage-dependent growth of control (solid symbols) and ODC over-expressing (open symbols) MCF-10 breast epithelial cells. *(A)* Influence of serum. pLXSN- and pLOSN-infected cells were plated at a density of 4×10^4 cells/35-mm dish in the presence of full growth medium. pLOSN-infected cells had been maintained in DFMO (0.1 mM) and washed off the drug 3 days prior to plating. Twenty-four hours after plating (time 0), duplicate dishes of control cells and of ODC-over-expressing MCF-10A cells were harvested for determination of plating efficiency, which was found to be similar for both cell types. Subsequently, the culture conditions were maintained in full growth medium (solid line) or were changed to serum-free medium (dotted line). Duplicate dishes were harvested at the times indicated, and the cells were counted by Coulter Counter. ODC activity measured in parallel duplicate dishes in growth medium was 1.42 nmol/mg and 356 nmol/mg in pLXSN- and pLOSN-infected cells respectively. *(B)* Effect of EGF (10 ng/ml) administration. Experimental details are as described above, except that the cells were always maintained in full growth medium, in the presence (solid line) and absence (dotted line) of EGF. ODC activity measured in parallel duplicate dishes (in the presence of EGF) was 1.51 nmol/mg and 363 nmol/mg in pLXSN- and pLOSN-infected cells respectively.

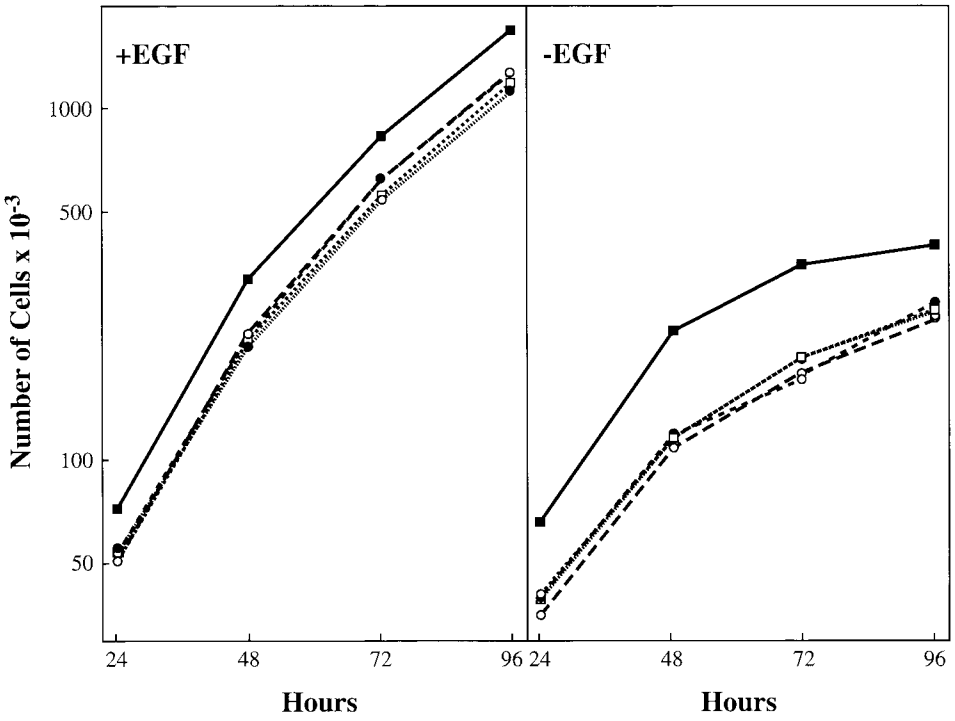

FIGURE 5 – Influence of variable degrees of ODC activity on the anchorage-dependent growth of MCF-10A breast epithelial cells in the presence and in the absence of EGF. pLOSN-infected MCF-10A cells maintained chronically in DFMO (0.1 mM) were washed off the drug. Three days later, they were plated in the absence or in the presence of the following concentrations of DFMO: 15 μM, 25 μM and 50 μM. The cell lines are indicated by the various dotted lines, which are almost superimposed; control, pLXSN-infected cells are indicated by the solid line. All cells were plated at a density of 4×10^4 cells/35-mm dish in regular growth medium in the presence or absence of EGF (10 ng/ml), as indicated. The data represent means of 2 separate experiments, each done in triplicate (n = 6 for each data point). The ODC activity of the various cell lines tested is shown in Table II.

TABLE II – LEVELS OF ODC ACTIVITY IN CONTROL AND ODC-OVER-EXPRESSING MCF-10A CELLS[1]

Cell line	ODC activity (nmol/mg protein)	
	+EGF	−EGF
neo	0.780	0.322
ODC, 0 DFMO	463	184
ODC, 15 μM DFMO	16.99	1.28
ODC, 25 μM DFMO	11.06	1.25
ODC, 50 μM DFMO	6.18	0.948

[1]Experimental conditions are as described in the legend to Figure 5. Parallel 100-mm dishes plated concomitantly with one of the 2 growth studies were harvested at the end of the experiment (96 hr) for determination of ODC activity.

more importantly, of epithelial cells (Clifford *et al.*, 1995). A direct role of polyamines in mammary carcinogenesis has not yet been demonstrated. However, the finding that breast-cancer specimens have considerably higher levels of polyamines than the surrounding normal breast tissue (Persson and Rosengren, 1989) suggests the possible involvement of these compounds in the development of mammary tumors.

To directly test the role of polyamine biosynthetic activity in mammary carcinogenesis, we induced ODC over-expression in the immortalized but otherwise normal MCF-10A human mammary epithelial cell lines. As reported for MCF-7 breast-cancer cells (Manni *et al.*, 1995*c*), it was necessary to expose ODC-infected MCF-10A cells to chronic DFMO treatment in order to maintain stable high levels of cellular enzymatic activity. In these experimental conditions, we observed that high ODC activity was due to over-expression of the infected gene rather than to amplification of the endogenous ODC (Fig. 1). Analysis of cellular polyamine profiles revealed that, as a result of ODC over-expression, there was selective accumulation of putrescine without significant changes in spermidine and spermine levels. The lack of increase in the cellular content of the 2 distal polyamines is likely to be due to the compensatory decrease in SAMDC (the enzyme directly regulating spermidine and spermine synthesis) and the increase in SSAT, which mediates back-conversion of spermidine and spermine to putrescine. In view of the polyamine profile induced by ODC over-expression, our experimental conditions may have underestimated the involvement of polyamine in mammary carcinogenesis, since increased levels of spermidine and spermine (in addition to putrescine) have been found in breast-cancer specimens, as compared with surrounding normal mammary tissue (Persson and Rosengren, 1989). We have successfully transfected MCF-7 breast-cancer cells with a SAMDC cDNA, which induced an increase in cellular spermine levels (Manni *et al.*, 1995*a*). We plan to use a similar experimental approach to test the role of this polyamine in mammary tumorigenesis.

The intracellular concentration of the polyamines is highly regulated by modulation of the enzymes involved in their synthesis and their catabolism. Such potent homeostatic mechanisms limit the polyamine pool size in eukaryotic cells, to prevent the cytotoxicity resulting from accumulation of these compounds (Tome *et al.*, 1994). On the other hand, the cellular levels of polyamines and their biosynthetic enzymes are higher in cancer cells, including breast-cancer cells, than in the surrounding normal tissue (Persson and Rosengren, 1989). In the aggregate, these findings emphasize that the cellular effects of polyamines are highly dose-dependent, probably within a relatively narrow range. Therefore, in investigating the role of ODC activation on mammary

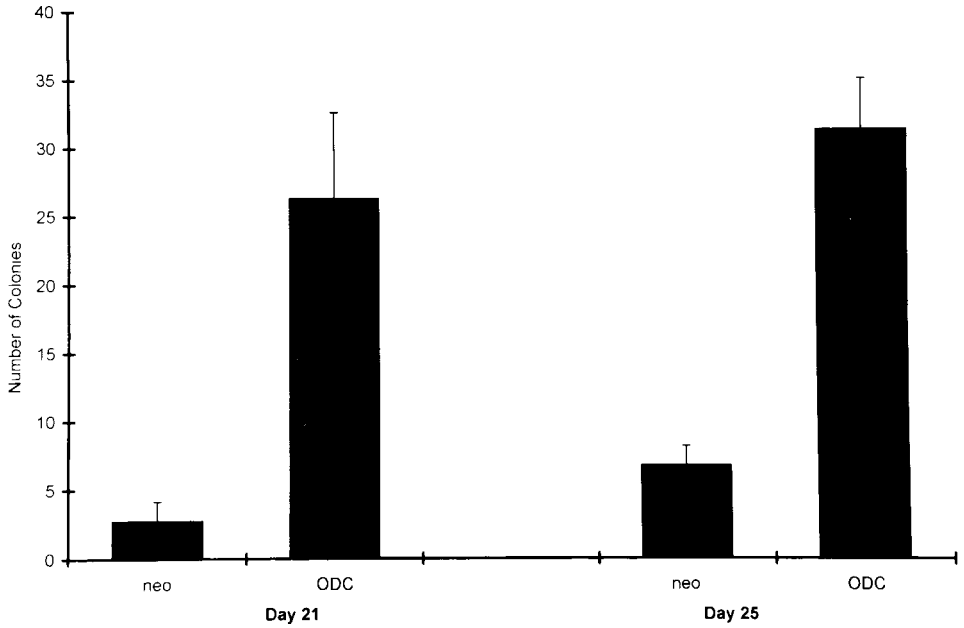

FIGURE 6 – Clonogenicity in soft agar of control (neo) and ODC-over-expressing MCF-10A cells (ODC). pLOSN-infected cells had been maintained in DFMO (0.1 mM) for approximately one month. The drug was removed 9 days before plating in soft agar. ODC activity measured at the time of plating was 0.967 ng/ml and 11.18 nnmol/mg in neo- and ODC-infected cells respectively. Bars represent the means ± SD of 3 replicate dishes per experimental condition.

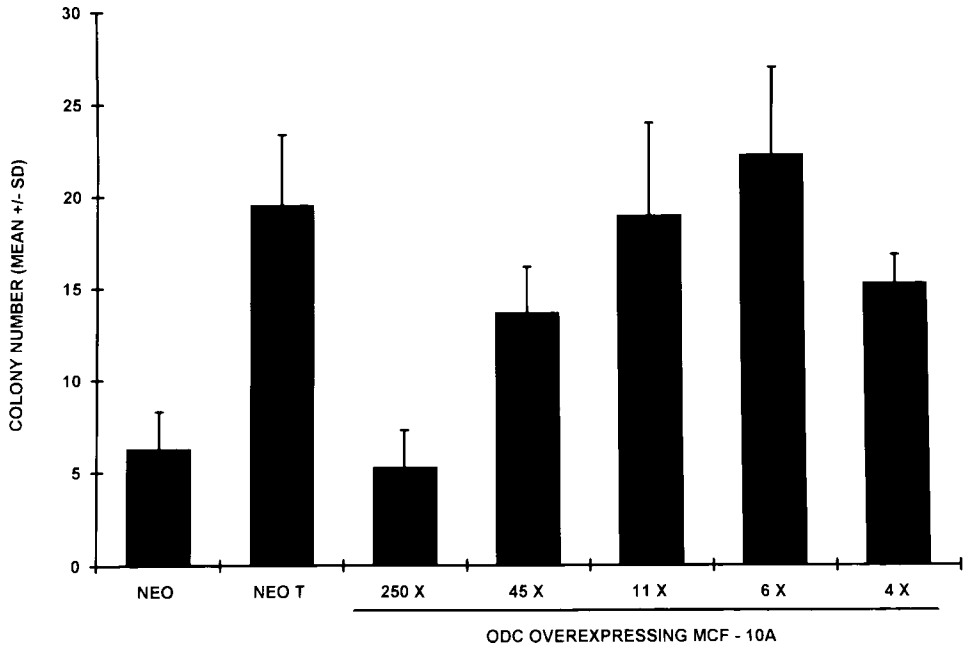

FIGURE 7 – Influence of different degrees of ODC over-expression on clonogenicity of MCF-10A cells. To generate cells with different degrees of ODC activity, pLOSN-infected MCF-10A cells maintained chronically in DFMO (0.1 mM) were washed off the drug. Three days later, they were plated in soft agar in the absence and in the presence of increasing concentrations of DFMO (5, 15, 25, 50 μM). The corresponding -fold excess in ODC activity over pLXSN-infected cells (neo) expressed by these clones is indicated in the figure. ODC activity was measured in parallel duplicate 100-mm dishes per clone plated simultaneously with the soft-agar assay. The partially transformed MCF-10A cells containing a mutated c-Ha-*ras* oncogene (neoT) were also plated in soft agar for comparison. The bars represent the means ± SD of 3 replicate dishes per experimental condition.

carcinogenesis, it was important to generate clones of MCF-10A cells with variable degrees of ODC activity. Our results, indeed, show that moderate but not marked degrees of ODC over-expression in MCF-10A cells conferred clonogenicity in soft agar, a conventional feature of the transformed phenotype. Such an effect on clonogenicity provided by ODC over-expression was comparable with that produced by oncogenic *ras* (Fig. 7). Thus, activation of polyamine biosynthesis may be one of the multiple genetic

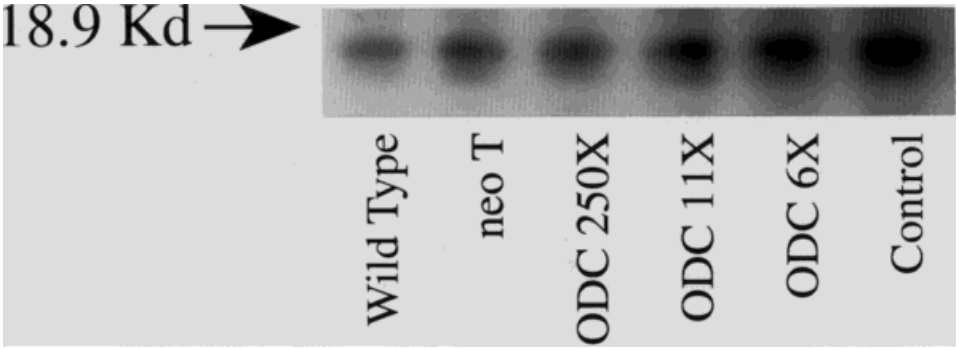

FIGURE 8 – ERK-2-kinase activity measured in cell extracts of wild-type, *ras* -transformed (neoT) and ODC over-expressing MCF-10A cells (see ''Material and Methods'' for technical details). Briefly, cell lysates were immunoprecipitated with anti-ERK-2 anti-serum (Santa Cruz Biotechnology) and the immunoprecipitates were subjected to an *in vitro* kinase assay using myelin basic protein as a substrate. The reaction mixture was resolved on a 15% SDS-PAGE and the phosphorylated myelin basic protein was visualized by autoradiography. The degree of enzymatic over-expression over neo-infected cells is indicated in the figure. Densitometric readings in the clones were as follows: wild-type, 1.22; neoT, 2.59; ODC 250×, 2.69; ODC 11×, 4.76; ODC 6×, 7.30; control (rat fibroblasts), 8.59.

events causally involved in mammary carcinogenesis. It is worth emphasizing that ODC-over-expressing MCF-10 cells manifested decreased anchorage-dependent growth under multiple experimental conditions, including the presence and the absence of serum and the presence and the absence of EGF. Furthermore, no dose-dependent effects of ODC on this biologic parameter were found. This finding indicates that the increased clonogenicity in soft agar is not a non-specific consequence of a growth advantage provided by ODC over-expression, but reflects the specific involvement of polyamines in transformation.

The mechanisms by which ODC over-expression leads to malignant transformation remain largely unknown. Control of protein phosphorylation may be a critical event (Hölttä *et al.,* 1994). ODC-transfected NIH-3T3 cells have increased tyrosine phosphorylation of the $pp60^{v\text{-}src}$ substrate p130 (Hölttä *et al.,* 1994). Furthermore, inhibitors of tyrosine kinases reversed the ODC-induced transformation in this system (Hölttä *et al.,* 1994). Our experiments tested the hypothesis that ODC over-expression influences the MAPK kinase-signalling cascade, a final common pathway activated by various growth factors, tumor promoters and differentiating agents. The importance of this pathway in malignant transformation has been emphasized by the finding that expression of constitutively active MAPK mutants increased AP-1-regulated transcription and led to morphologic transformation of mammalian cells (Mansour *et al.,* 1994). Furthermore, several lines of mainly unpublished evidence (summarized in Clark and Der, 1995) support the possibility that de-regulation of this signalling transduction system may be involved in breast-cancer development. Our data demonstrate an interaction between the polyamines and the MAPK-cascade pathways, and suggest that activation of the latter may mediate ODC-induced clonogenicity of MCF-10A cells. In support of this possibility is the observed parallelism between the dose-dependent effects of ODC expression on clonogenicity (Fig. 7) and ERK-2 kinase activity (Fig. 8). We are aware that this correlative finding does not establish a cause-effect relationship between ODC-stimulated activation of ERK-2 kinase and the induction of clonogenicity. Additional studies using dominant negative mutants of ERK-2 (as well as other kinases of the MAPK-signalling cascade) will be necessary to establish the role of these signalling elements in ODC-induced transformation of mammary epithelial cells.

In summary, our findings indicate that activation of the polyamine pathway directly contributes to the acquisition of the transformed phenotype by human mammary epithelial cells. Our data also show that the effect of ODC over-expression on transformation is dissociated from that on proliferation, and is likely to be mediated through activation of the MAPK-cascade pathway. We are currently testing the influence of ODC over-expression in MCF-10A cells on tumorigenesis in nude mice, as well as its possible cooperativity with other oncogenic signals involved in breast-cancer development.

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