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# Basic Fibroblast Growth Factor Selectively Regulates Ornithine Decarboxylase Gene Expression in Malignant H-*ras* Transformed Cells

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**Abstract** Cell growth regulation by fibroblast growth factors (FGFs) is highly complex. The present study demonstrates a novel link between alterations in bFGF regulation during malignant conversion and the expression of ornithine decarboxylase, a key rate-limiting and regulatory activity in the biosynthesis of polyamines. H-*ras* transformed mouse 10T½ cell lines exhibiting increasing malignant potential were investigated for possible bFGF-mediated changes in ornithine decarboxylase gene expression. Selective induction of ornithine decarboxylase gene expression was observed, since, in contrast to nontransformed 10T½ cells and cells capable of only benign tumor formation, H-*ras* transformed metastatic cells exhibited marked elevations in ornithine decarboxylase message levels. Evidence for regulation of ornithine decarboxylase gene expression by bFGF at both transcription and posttranscription was found. Actinomycin D pretreatment of malignant cells prior to bFGF exposure inhibited the increase in ornithine decarboxylase message. Furthermore, striking differences in the rates of ornithine decarboxylase message decay were observed when cells treated with bFGF were compared to untreated control cells, with the half-life of ornithine decarboxylase mRNA increasing from 2.4 h in untreated cells to 12.5 h in cells exposed to bFGF. Evidence was also obtained for a cycloheximide-sensitive regulator of ornithine decarboxylase gene expression whose effect, in combination with bFGF, resulted in a further augmentation of ornithine decarboxylase gene expression. Furthermore, evidence is presented to suggest a possible role for G-protein–coupled events in the bFGF-mediated regulation of ornithine decarboxylase gene expression. The bFGF regulation of ornithine decarboxylase expression in H-*ras* transformed malignant cells appeared to occur independent of protein kinase C–mediated events. These results show that bFGF can modulate ornithine decarboxylase gene expression in malignant H-*ras* transformed cells and further suggests a mechanism of growth factor stimulation of malignant cells wherein early alterations in the regulatory control of ornithine decarboxylase gene expression are critical. 

**Key words:** basic fibroblast growth factor, ornithine decarboxylase, H-*ras* transformed cells, G-protein, protein kinase C

Growth factors are fundamentally involved in cellular processes that are important in the progression of malignant disease [Sporn and Todaro, 1980; Heldin and Westermark, 1984; Cross and Dexter, 1991; Wright et al., 1990, 1993]. Two growth factor families, transforming growth factor-β (TGF-β) and fibroblast growth factor (FGF) are useful models for examining the involvement of growth factors in mechanisms of malignant progression [Wright et al., 1993]. TGF-β appears to be a fundamental multifunctional regulator of cellular behavior. A role for TGF-$\beta_1$ autocrine regulation of DNA synthesis and cell proliferation in malignancy has been suggested [Hurta et al., 1991, 1993; Schwarz et al., 1988, 1990]. In this regard, we have previously demonstrated a link between TGF-$\beta_1$ regulation of cellular transformation and alterations in the expression of ribonucleotide reductase, a highly controlled rate-limiting step in DNA synthesis [Hurta et al., 1991]. Furthermore, these studies were extended to demonstrate a role for TGF-$\beta_1$ in the regulation of ornithine decarboxylase gene expression in malignant H-*ras* transformed cells [Hurta et al., 1993]. Ornithine decarboxylase is the first and rate-limiting enzyme in the synthesis of polyamines which are necessary for all animal, plant, and microbial cell proliferation and survival [Tabor and Tabor, 1984; Pegg, 1988; Hayashi, 1989], and recent studies have shown that altered ornithine decarbox-

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ylase regulation can play important roles in mechanisms of malignancy with NIH-3T3 fibroblasts [Moshier et al., 1993], in cells containing a mutated *ras* gene [Hibshoosh et al., 1991], and in premalignant epidermal cells [Clifford et al., 1995]. Similar to the TGF-β family, the FGFs appear to be multifunctional and possess the potential to participate in a variety of biological activities. Synergistic interactions between FGF and TGF-β have been observed [Wright et al., 1993; Pepper et al., 1990; Rizzino et al., 1986]. Basic fibroblast growth factor (bFGF) is synthesized by a variety of normal and tumor cells [Rifkin and Moscatelli, 1989], and observations of high levels of bFGF activity associated with cultured tumor cells is consistent with postulated roles for FGFs in mechanisms of malignant transformation as mediators of genetic instability important in malignant progression [Huang and Wright, 1994; Huang et al., 1994] and as mitogenic stimulators of tumor cell proliferation [Cross and Dexter, 1991; Rifkin and Moscatelli, 1989]. In this study, we have investigated the possible bFGF regulation of two proliferation linked enzymes, ribonucleotide reductase and ornithine decarboxylase, in several H-*ras* transformed cell lines exhibiting different malignant potential [Hurta et al., 1991, 1993; Egan et al., 1987].

## MATERIALS AND METHODS

### Cell Lines and Growth Conditions

Mouse cell lines were routinely cultured at 37°C on plastic tissue culture plates (Nunc Scientific) in α-minimal essential medium (α-MEM) (Flow Laboratories, Quebec) supplemented with antibiotics and 7% (v/v) fetal bovine serum (Intergen, New York, USA). Cells grew with doubling times of approximately 18–20 h. In experiments utilizing the growth factor bFGF (Gibco BRL) a serum-free medium was used which contained 4 μg/ml of transferrin (Sigma Chemical Co., St. Louis, MO) and 2 μg/ml of insulin (Sigma Chemical Co.) in 100 ml of α-MEM [Hurta et al., 1991; Schwarz et al., 1988]. In this medium, cells grew with doubling times of approximately 20–22 h. Cells were grown overnight in α-MEM with 7% fetal bovine serum and then switched to the serum-free medium for 18 h prior to exposure to 10 ng/ml bFGF at predetermined times [Hurta et al., 1991, 1993]. Cells were removed from the surfaces of tissue culture plates using a 0.3% buffered trypsin solution (Difco Laboratories, Michigan) [McClarty et al., 1987].

### Northern Blot Analysis

Total cellular RNA was prepared by a rapid extraction method [Gough, 1988] and subjected to electrophoresis through 1% formaldehyde-agarose gels followed by transfer to Nytran nylon membranes (Schleicher and Schuell, Keene, NH). Blots were prehybridized and hybridized as we have previously described [McClarty et al., 1990; Hurta and Wright, 1995]. Hybridization occurred in the presence of a $^{32}$P-labelled Pst-1–generated fragment of ODC cDNA from the pODC934 plasmid (provided by A.E. Pegg, Milton S. Hershey Medical Center, Hershey, PA) or in the presence of either a $^{32}$P-labelled Nco1-generated fragment containing the cDNA of clone 65 (R1) or the Pst 1–generated fragment containing the cDNA of clone 10 (R2) [Hurta et al., 1991]. Probes were labelled using an oligolabelling kit (Pharmacia LKB Biotechnology, Quebec) and α-$^{32}$P–labelled dCTP (Amersham, Ontario). Blots were washed and autoradiography was performed as previously described [Hurta et al., 1991; McClarty et al., 1990; Choy et al., 1989]. Loading was monitored with a plasmid containing the glyceraldehyde-3-phosphate dehydrogenase gene labelled by nick translation [Edwards et al., 1985] or with a 28s ribosomal RNA probe isolated from the plasmid $pA_{BB}$ by digestion with BamH 1 [Gonzalez et al., 1985]. Densitometric analyses of appropriate autoradiograms were carried out using a Beckman DU-8 gel scanning spectrophotometer and the one dimensional Bio-Rad program (Bio-Rad, Ontario).

### Assay for Ornithine Decarboxylase Activity

Ornithine decarboxylase enzyme activity was measured using a modification of the method of Russell and Snyder [1968] as described by Yoshida et al. [1992] and Hurta and Wright [1994]. Cell pellets were solubilized in 50 mM Tris-HCl (pH 7.5) containing 5 mM dithiothreitol, sonicated briefly, and then centrifuged at 10,000*g* to remove the insoluble fraction. Protein content of the supernatant was determined using a Bio-Rad protein assay kit, with bovine serum albumin as a standard. The enzyme assay mixture consisted of 0.4 mM L-ornithine, 0.125 μCi of DL-[1-$^{14}$C] ornithine (58 mCi/mmole) (Amersham), 0.02 mM pyridoxal phosphate, 0.4 mM EDTA, 50 mM Tris-HCl (pH 7.5), and cellular extract in a total volume of 0.52 ml. Incubations were carried out for 60 min at 37°C. The

reaction was terminated by addition of 0.8 ml of 2 M citric acid. Ornithine decarboxylase activity was determined by measuring the release of [$^{14}$C] $CO_2$, which was collected in filter paper soaked with 25% phenylethylamine. Radiactivity was determined by liquid scintillation spectroscopy using a model L57800 scintillation counter (Beckman, Mississauga, Canada).

## RESULTS

### Properties of H-ras Transfected Mouse Fibroblasts

Mouse 10T½ cells were transfected with plasmid pAL8A which contains T-24 H-*ras* and the neo$^R$ gene. Cell lines were established that were either morphologically transformed (C2, C3) or morphologically nontransformed (NR3 and NR4) [Schwarz et al., 1988, 1990; Egan et al., 1987]. MDS.R5 is a clone of radiation transformed 10T½ cells [Schwarz et al., 1988, 1990; Egan et al., 1987]. A summary of the biological characteristics of these cell lines is presented in Table I. It is important to note that the parental 10T½ cell line is not tumorigenic; the NR3 and MDS.R5 cell lines are capable of forming benign tumors in syngeneic hosts, while the NR4, C2, and C3 cell lines exhibit malignant characteristics.

### Selective Induction of Ornithine Decarboxylase Gene Expression by bFGF

The effects of bFGF on ODC gene expression in the cell lines shown in Table I are presented in Figure 1. Elevations in ODC mRNA levels were observed following bFGF treatment, but these elevations occurred only in the malignant NR4, C2, and C3 cell lines. An increase in ODC gene expression was not detected in either the bFGF-treated parental 10T½ cells or in the benign tumor cell line NR3 or the MDS R5 radiation transformed clone of 10T½ cells. Obvious alterations in ODC gene expression were observed in the highly malignant cells following bFGF treatment. Densitometric evaluation of appropriately exposed autoradiograms revealed 3.4-, 3.0-, and 3.2-fold increases, 3.4-, 5.3-, and 7.7-fold increases, and 7.5-, 7.1-, and 6.4-fold increases in ODC mRNA levels in NR4, C2, and C3, respectively, following exposure to bFGF (at 10 ng/ml) for 2, 4, and 8 h, respectively.

To determine if the increases in ODC message observed in bFGF-treated malignant cells lead to elevated enzyme activity, the levels of ODC activity in C3 cells were measured following growth factor treatment for 2, 4, and 8 h and compared to the activity obtained with nontransformed 10T½ cells. Interestingly, marked elevations in ODC enzyme activity were observed with growth factor–exposed C3 cells, which was time-dependent, and ranged from about fivefold following a 2 h exposure to bFGF to more than thirtyfold after 8 h of bFGF treatment (Fig. 2). This is in striking contrast to the results obtained with 10T½ cells, which showed only a modest 1.5-fold increase in enzyme activity at all three bFGF treatment times.

Previous studies have described TGF-$\beta_1$ and tumor promoter–mediated regulation of both

**TABLE I. Tumorigenic and Metastatic Properties of Mouse 10T½ Cell Lines***

| Cell Line | Tumorigenicity frequency | Experimental Metastases | | |
|---|---|---|---|---|
| | | Frequency | Number of Lung Nodules (mean ± S.E.) | Degree of malignancy |
| 10T½ | 0/12 | 0/12 | 0 | Normal |
| MDS.R5 | 5/5 | 0/6 | 0 | Benign |
| NR3 | 6/8 | 1/13 | 0.1 ± 0.1 | Benign |
| NR4 | 10/10 | 12/19 | 2.0 ± 0.5 | Metastatic |
| C2 | 11/11 | 8/8 | 118 ± 6 | Metastatic |
| C3 | 11/11 | 14/14 | 121 ± 20 | Metastatic |

*The data in this table was summarized from previously reported observations (Egan et al., 1987). Tumorigenicity was determined following subcutaneous injections of $3 \times 10^5$ cells in syngeneic C3H/HeN mice except for the MDS.R5 line, where $10^6$ cells were injected. Latency was based on six mice with detectable tumors. Subcutaneous injection of up to $10^7$ control 10T½ cells into either syngeneic C3H/HeN or immunodeficient BALB/c nu/nu mice did not result in tumor formation. Metastatic potential was determined by the experimental metastasis assay with a tumor cell innoculum of $3 \times 10^5$ cells injected in a 0.2 ml volume into the tail vein of mice. Cells were trypsinized from subconfluent cultures, washed, and adjusted to the appropriate concentration in Hanks' balanced salt solution. Recipient animals were sacrificed 21 days later by ether anaesthesia and Bouin solution injected intratracheally. Stained lungs were then removed, and metastatic foci were counted under a dissecting microscope.

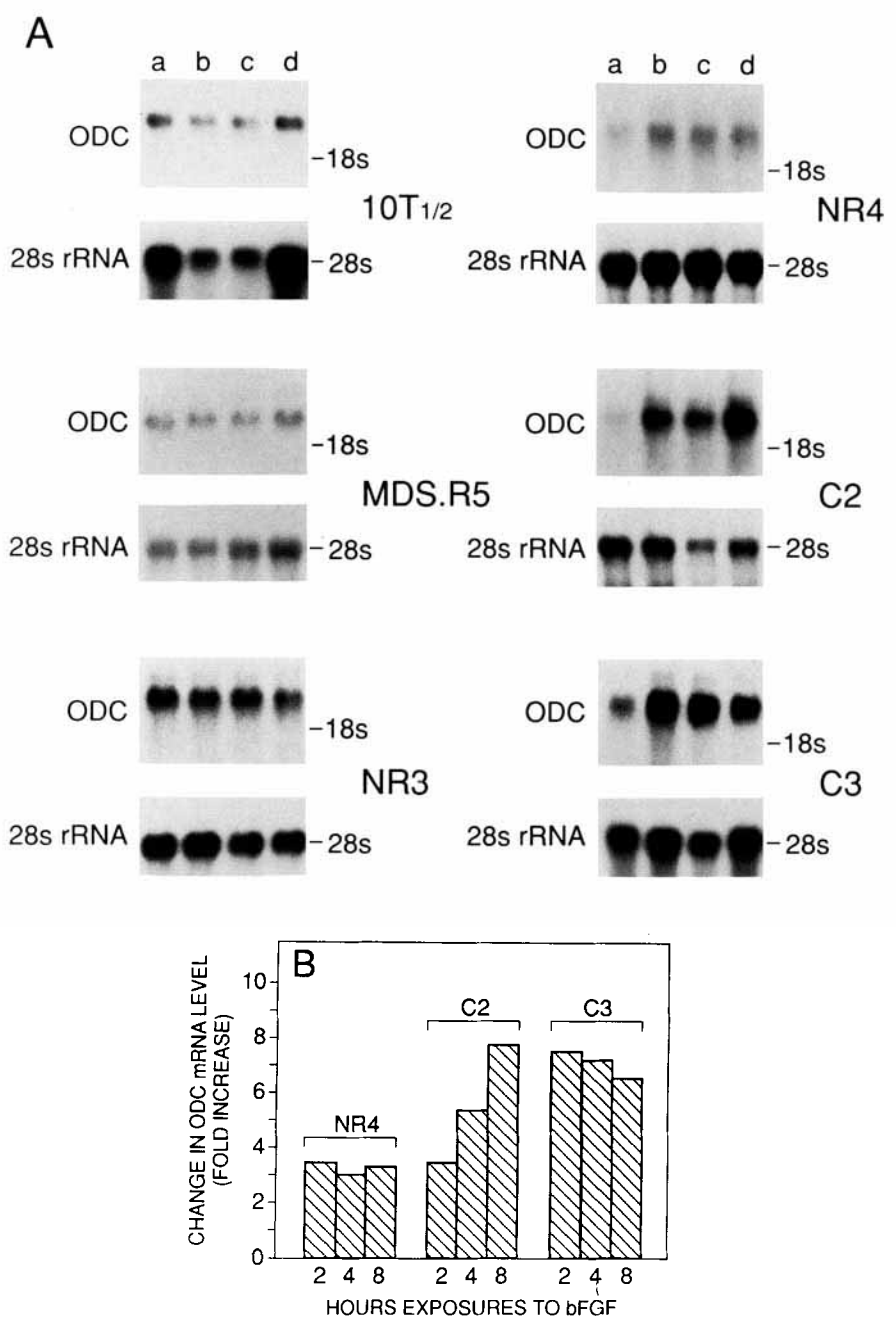


**Fig. 1.** **A:** Northern blot analysis of ODC mRNA levels following exposure to bFGF (10 ng/ml). Increased ODC mRNA was noted only in the metastatic NR4, C2, and C3 cell lines. Cells were exposed to bFGF for 2 (*lane b*), 4 (*lane c*), and 8 (*lane d*) h, respectively. Control cells were grown in the absence of bFGF; these mRNA levels are shown (*lane a*). RNA loading was determined with a 28s rRNA cDNA for each of the blots shown. The 10T½ and MDS.R5 ODC and corresponding 28s rRNA autoradiograms were exposed for 1 week and 1 h, respectively, the NR3 ODC and corresponding 28s rRNA autoradiograms were exposed for 96 h and 1 h, the NR4 and C2 ODC and corresponding 28s RNA autoradiograms were exposed for 48 h and 1 h, the C3 ODC and corresponding 28s rRNA autoradiograms were exposed for 24 h and 1 h, respectively, at −70°C with intensifying screens. **B:** The data obtained by densitometry is shown for the NR4, C2, and C3 cell lines, which exhibited increases in ODC mRNA expression following exposure to bFGF.

ornithine decarboxylase and ribonucleotide reductase [Hurta et al., 1991, 1993; Choy et al., 1989; Hurta and Wright, 1992, 1994]. Mammalian ribonucleotide reductase plays a key role in the synthesis of DNA, since it is responsible for the de novo reduction of ribonucleoside diphosphates to their corresponding deoxyribonucleoside diphosphate forms required for DNA synthesis [Wright, 1989]. The enzyme consists of two dissimilar protein components, often called R1

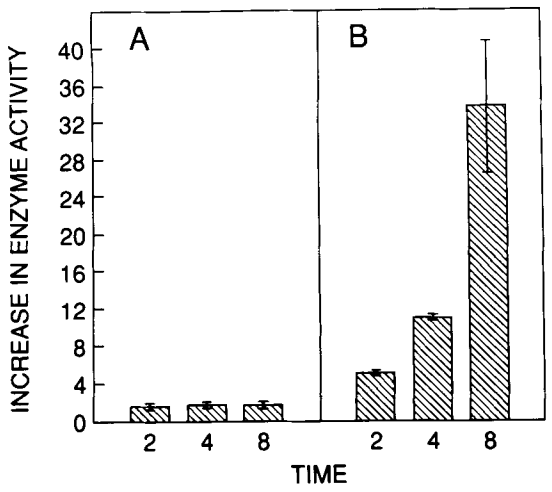


Fig. 2. ODC enzyme activity levels in cells treated with bFGF for various times relative to ODC enzyme activity in untreated cells. Mouse 10T½ nontransformed cells (**A**) or malignant H-*ras* transformed C3 cells (**B**) were treated with bFGF (10 ng/ml) for 2, 4, or 8 h as indicated, and enzyme activity was measured. The results ± standard errors are from two determinations for 10T½ and three determinations for C3 cells. The levels of ODC activity in untreated 10T½ and C3 cells were similar and ranged between 0.25 and 0.28 nmoles $CO_2$/h/mg protein in these experiments.

and R2, which are noncoordinately regulated [Wright, 1989]. Therefore, the effect of bFGF treatment on the expression of ribonucleotide reductase R1 and R2 mRNA levels in C3 cells was determined. Figure 3 shows that no increase in either R1 or R2 message levels was detected. Also, no increase in either R1 or R2 message levels was detected in either 10T½ or NR3 cells following bFGF treatment (data not shown).

### Effect of bFGF Treatment on Transcription of the Ornithine Decarboxylase Gene

The possibility that the elevations in ODC message observed in malignant C3 cells following exposure to bFGF were due to changes in gene transcription rates was examined by pretreating C3 cells with the transcription blocker actinomycin D [Phillips and Crowthers, 1986; Olson and Spizz, 1986] prior to bFGF treatment. As shown in Figure 4, actinomycin D inhibited the elevation in ODC message previously observed following exposure of these cells to bFGF. In the absence of actinomycin D, 6.5- and 5.9-fold increases in ODC mRNA levels were determined at 1 and 3 h exposure to bFGF, respectively (Fig. 4, lanes b,c). In the presence of the transcription blocker, no increase in ODC mRNA levels was detected following treatment

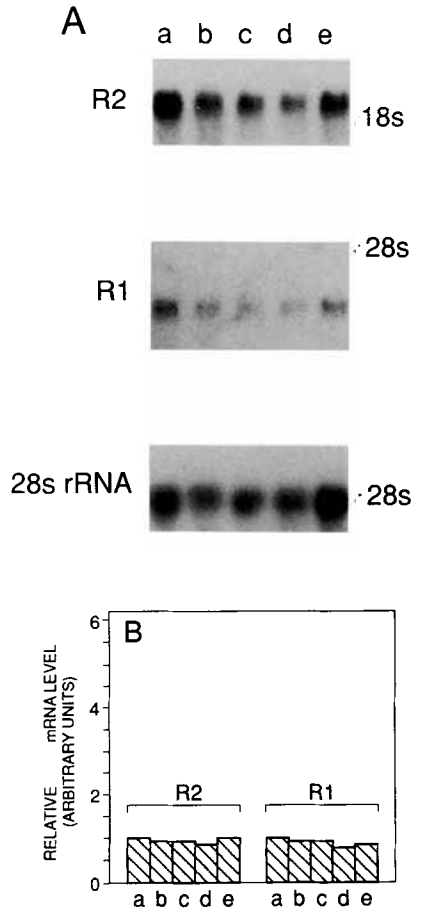


Fig. 3. **A:** Northern blot analysis of ribonucleotide reductase R2 and R1 mRNA levels in C3 cells following exposure to bFGF (10 ng/ml). Northern blots of R2 mRNA, R1 mRNA, and 28s rRNA levels (as loading control) in C3 cells cultured in the absence (*lane a*) or in the presence of bFGF (10 ng/ml) for 2 h (*lane b*), 4 h (*lane c*), 8 h (*lane d*), and 24 h (*lane e*), respectively, are shown. Autoradiograms for R2, R1, and 28s rRNA expression were exposed for 24, 96, and 2 h, respectively, at −70°C with intensifying screens. **B:** The data obtained by densitometry is shown.

of the cells with bFGF (Fig. 4, lanes e,f). Inhibitors are commonly used to investigate questions of gene expression control at the level of transcription [Phillips and Crowthers, 1986; Olson and Spizz, 1986; Hurta and Wright, 1994, 1995; Yang and Yang, 1994], and, although this approach is somewhat indirect, the findings obtained in this study suggest that the bFGF effects on ODC gene expression are mediated, at least partly, by changes in the transcription process.

### Effect of Protein Synthesis Inhibition on Ornithine Decarboxylase mRNA Induction by bFGF

To determine whether or not the bFGF-mediated elevations in ODC mRNA determined

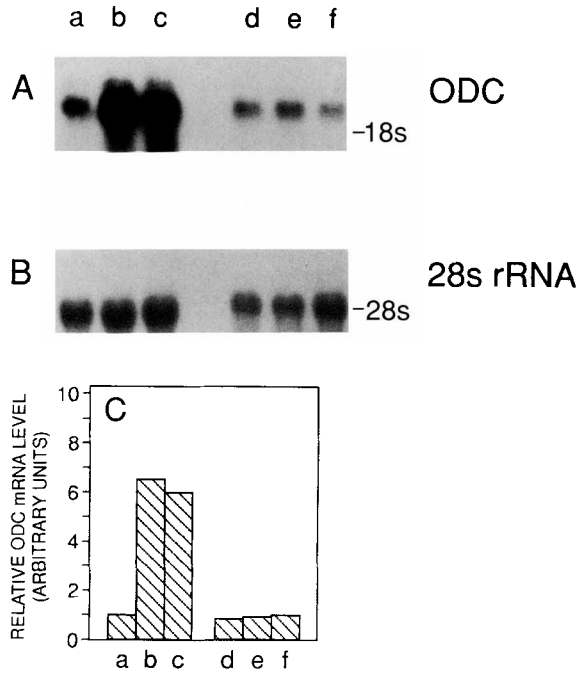


**Fig. 4.** **A,B:** Northern blot analysis of ODC mRNA levels. C3 cells (control) (*lane a*); C3 cells exposed to bFGF (10 ng/ml) for 1 h (*lane b*) and 3 h (*lane c*) and in the presence of 5 μg/ml actinomycin D without bFGF (*lane d*); in the presence of 5 μg/ml actinomycin D with bFGF (10 ng/ml) for 1 h (*lane e*) and 3 h (*lane f*), respectively. Panel A is an autoradiogram of ODC mRNA expression exposed for 72 h; whereas panel B is a 2 h exposure of a 28s rRNA autoradiogram as a loading control. All autoradiograms were exposed at −70°C with intensifying screens. **C:** The data obtained by densitometry is shown.

in C3 cells required protein synthesis, the effects of the protein synthesis inhibitor cycloheximide [Hurta and Wright, 1992; Le et al., 1992; McClarty et al., 1986] on bFGF-mediated alterations of ODC gene expression were investigated with C3 cells. Figure 5 shows that cycloheximide treatment (10 μg/ml) alone markedly elevated ODC message levels in C3 cells. A sixfold increase in ODC mRNA levels was found when C3 cells were treated with cycloheximide alone (Fig. 5, lane c). This observation is consistent with previous studies showing that cycloheximide treatment can elevate ODC mRNA levels in some mammalian cells [Olson and Spizz, 1986]. This effect appears to be cell type–specific since a reduction in ODC message levels in cycloheximide-treated cells has also been reported [Katz and Kahana, 1987]. Figure 5 also illustrates that the elevation in ODC mRNA levels observed in the presence of cycloheximide can be further increased when C3 cells are exposed to cycloheximide and bFGF together. Following exposure of C3 cells to cycloheximide and bFGF for 1 h, a 11.7-fold elevation in ODC mRNA levels was observed (Fig. 5, lane d). In the absence of cycloheximide treatment, a 3.8-fold elevation in ODC mRNA levels was noted in C3 cells following a 1 h exposure to bFGF (Fig. 5, lane b). These observations suggest that ODC gene expression in the highly malignant C3 cell line is controlled in a positive manner by protein synthesis inhibition following cycloheximide treatment.

### Stability of Ornithine Decarboxylase Message in Malignant C3 Cells Following Exposure to bFGF

Although bFGF-mediated elevations of ODC mRNA levels may occur via changes in the transcriptional process (Fig. 4), growth factors can also regulate gene expression posttranscriptionally through alterations in message stability [Amara et al., 1995; Ross, in press]. Therefore, the possibility that changes at the posttranscrip-

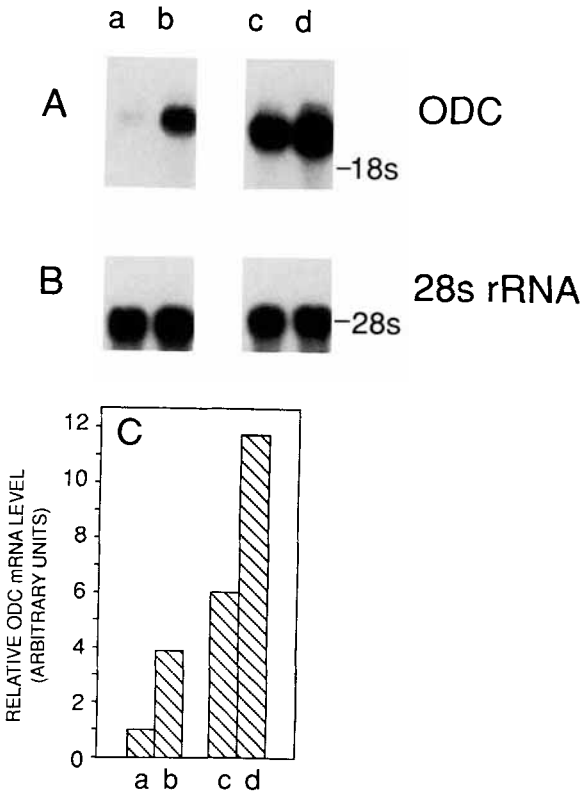


**Fig. 5.** **A,B:** Northern blot analysis of ODC mRNA levels. C3 cells (control) (*lane a*); C3 cells exposed to bFGF (10 ng/ml) for 1 h (*lane b*) and in the presence of cycloheximide (10 μg/ml) and the absence of bFGF (*lane c*); C3 cells in the presence of cycloheximide (10 μg/ml) and bFGF (10 ng/ml) for 1 h (A, *lane d*). Panel A is an autoradiogram of ODC mRNA expression exposed for 24 h, whereas panel B is a 2 h exposure of a 28s rRNA autoradiogram as a loading control. All autoradiograms were exposed at −70°C with intensifying screens. C3 control cells were grown in the absence of bFGF and cycloheximide. **C:** The data obtained by densitometry is shown.

tional level in response to bFGF also occur was investigated. Since a common mechanism for regulating message levels posttranscriptionally takes place via alterations in the decay rates of mature message in response to external stimuli [Chen et al., 1993; Amara et al., 1994; Ross, in press], the rate of decay of the mature ODC message was evaluated in untreated and in bFGF-treated C3 cells after exposure to an inhibitor of transcriptional initiation, 5,6-dichloro-1-β-D ribofuranosylbenzimidazole (DRB) [Mukherjee and Molloy, 1987; Hurta and Wright, 1995]. As shown in Figure 6, ODC mRNA in C3 cells is markedly more stable following bFGF treatment. Assuming that the decay of ODC mRNA after blocking RNA synthesis follows with first-order kinetics, the half-life of ODC mRNA in bFGF-treated cells, estimated by extrapolation, was increased approximately by five-fold over that found in cells not treated with bFGF. The half-life of ODC message in untreated C3 cells was estimated to be 2.4 h, whereas the half-life of this message in bFGF-treated cells was dramatically altered and increased to approximately 12.5 h. These findings indicate that bFGF is capable of regulating ODC mRNA levels through a mechanism of posttranscriptional stabilization.

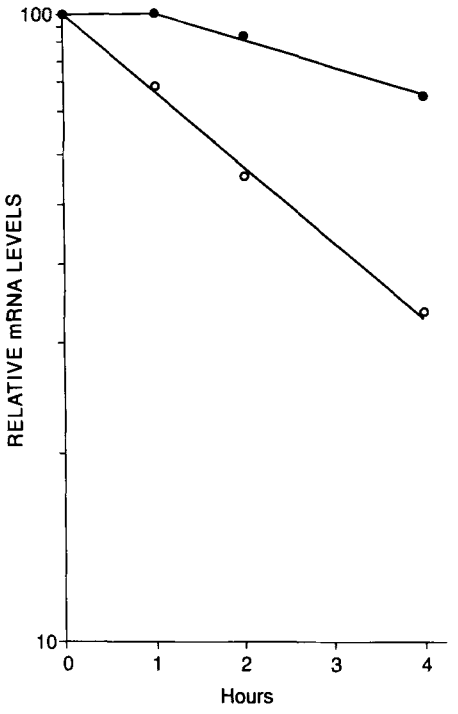


**Fig. 6.** Stability of ODC mRNA in untreated (○) and bFGF-treated C3 cells (●). C3 cells which were exposed to bFGF (10 ng/ml) (treated cells) or to an aqueous solution with 0.1% bovine serum albumin (vehicle within which bFGF was prepared) (untreated cells) for 3 h were subsequently treated with 5,6-dichloro-1-β-D ribofuranosylbenzimidazole (63 μM). Total RNA was isolated at the times indicated and subjected to Northern blot analysis as described. The relative levels of ODC mRNA were determined by densitometric evaluation of autoradiograms exposed in the linear range for each set of samples. The results presented are from duplicate determinations.

### Possible Involvement of G-Proteins in bFGF-Regulated Ornithine Decarboxylase Gene Expression

Previous studies have shown that guanine nucleotide binding proteins (G-proteins) can participate in transducing the mitogenic signal of polypeptide growth factors [Chambard et al., 1987; Hurta and Wright, 1995], and pertussis toxin is known to inhibit the activity of several G-proteins via their ADP ribosylation [Katada et al., 1984]. To test the possibility of G-protein involvement in the bFGF modulation of ODC gene expression, C3 cells were pretreated with pertussis toxin alone or in combination with bFGF. Total cellular RNA was isolated, and ODC gene expression was evaluated by Northern blot analysis (Fig. 7). Treatment of C3 cells with bFGF (10 ng/ml) alone for 2 h resulted in a 5.6-fold elevation of ODC mRNA (Fig. 7, lane b). Treatment of C3 cells with pertussis toxin (100 ng/ml) alone did not alter the level of ODC expression (Fig. 7, lane c). However, bFGF-induced elevations of ODC gene expression were markedly reduced in C3 cells exposed to pertussis toxin. Only an approximately 1.2-fold elevation in ODC mRNA levels was noted following bFGF exposure (2 h) to cells pretreated with pertussis toxin (100 ng/ml) for 3 h (Fig. 7, lane d). This observation suggests a possible role(s) for a pertussis toxin–sensitive G-protein(s) involvement in the bFGF-modulated upregulation of ODC gene expression in these metastatic H-*ras* transformed cells.

### Effect of Protein Kinase C Inhibitors on ODC mRNA Accumulation Following bFGF Exposure

The bFGF transduction pathway involves activation of its receptor tyrosine kinase activity [Lee et al., 1989; Coughlin et al., 1988], but it can also involve protein kinase C (PKC) activation [Presta et al., 1989]. The possibility that the accumulation of ODC mRNA caused by bFGF is mediated by protein kinase C–regulated events was examined using the highly malignant C2 and C3 cell lines. Previous studies

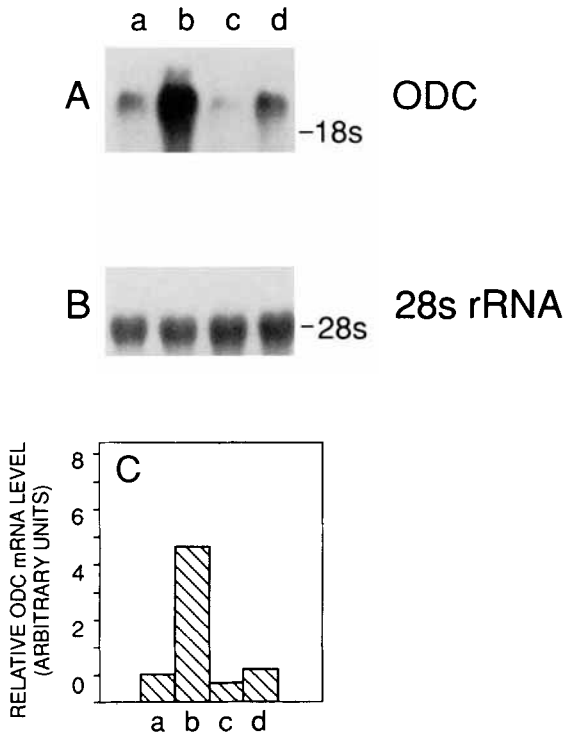


**Fig. 7.** **A,B:** Effect of bFGF on ODC mRNA expression, with or without pertussis toxin pretreatment. Northern blot analysis of ODC expression in C3 cells. C3 cells (control cells) (*lane a*), C3 cells exposed to bFGF (10 ng/ml) for 2 h (*lane b*), C3 cells treated with pertussis toxin (100 ng/ml) for 3 h, and then pertussis toxin treatment was continued without (*lane c*) or with (*lane d*) bFGF (10 ng/ml) for an additional 2 h. The ODC (A) and 28s rRNA (B) autoradiograms were exposed at −70°C with intensifying screens for 24 h and 1 h, respectively. **C:** The data obtained by densitometry is shown.

have shown that the biological properties including the malignant characteristics of these two cell lines are very similar (Table I). C2 cells were exposed to bFGF (10 ng/ml) in the absence or presence of H7 and HA1004, which are inhibitors of varying specificity for protein kinase C. H7 has been reported to be a potent and selective PKC inhibitor with a $K_i$ of 6 μM, and HA1004 inhibits PKC with a $K_i$ of 40 μM [Hidaka et al., 1984]. Neither H7 nor HA1004 treatment of C2 cells abrogated the bFGF induction of ODC mRNA expression (Fig. 8A). Also, neither H7 nor HA1004 alone altered ODC mRNA levels (Fig. 8A). bFGF (10 ng/ml) alone caused a 4.7-fold elevation in ODC mRNA expression (Fig. 8A, lane b). Exposure of cells to either H7 (50 μM) or HA1004 (50 μM) in combination with bFGF (10 ng/ml) resulted in 4.7- and 5.4-fold increases in ODC mRNA expression, respectively (Fig. 8A, lanes d,f). The role of PKC-mediated events in the induction of ODC expression by bFGF was also examined after downregulation of PKC activity with prolonged exposure to 12-0-tetradecanoylphorbol-13-acetate (TPA) [Choy et al., 1989; Young et al., 1987]. In these studies, shown in Figure 8B, C3 cells were pretreated with 0.1 μM TPA for 48 h and then exposed to bFGF (10 ng/ml) for 1 h. Exposure of nonpretreated C3 cells to bFGF resulted in a 3.8-fold increase in ODC mRNA levels (Fig. 8B, lane b). Notably, in C3 cells pretreated with TPA for 48 h, a similar increase (3.7-fold) in ODC mRNA levels following exposure to bFGF was observed (Fig. 8B, lane d). These results suggest that bFGF (10 ng/ml) exerts its action on ODC expression in C2 and C3 cells, which are both highly metastatic cell lines, through a protein kinase C–independent pathway.

## DISCUSSION

Ornithine decarboxylase catalyzes the conversion of ornithine to putrescine and is the first and rate-controlling enzyme in the synthesis of polyamines. ODC is controlled by a variety of agents capable of stimulating cellular proliferation, and ODC gene expression is subject to complex regulation that can occur at transcriptional, translational, and posttranslational levels [Kontula et al., 1984; Kahana and Nathans, 1985; Persson et al., 1985; Hovis et al., 1986; Sertich and Pegg, 1987; Holtta and Pohjanpelto, 1986; Fong et al., 1976]. The present study provides evidence of an intriguing link between alterations in bFGF responses during malignant conversion and the expression of ODC. Increased ODC gene expression following bFGF exposure occurred in H-*ras* transformed cells that exhibited malignant characteristics, namely NR4, C2, and C3 cells. Normal cells or cells capable of only benign tumor formation did not show these changes. These observations resemble studies of TGF-$\beta_1$ regulation of ornithine decarboxylase gene expression in normal and malignant cells [Hurta et al., 1993], which showed that malignant *ras* transformed cells contain alterations in signal transduction pathways that lead to novel regulation of this growth-related activity. Interestingly, under conditions in which ODC gene expression was altered by bFGF exposure in this study, bFGF did not alter the expression of ribonucleotide reductase genes in either normal cells or tumorigenic/metastatic cells. This finding is quite different from the

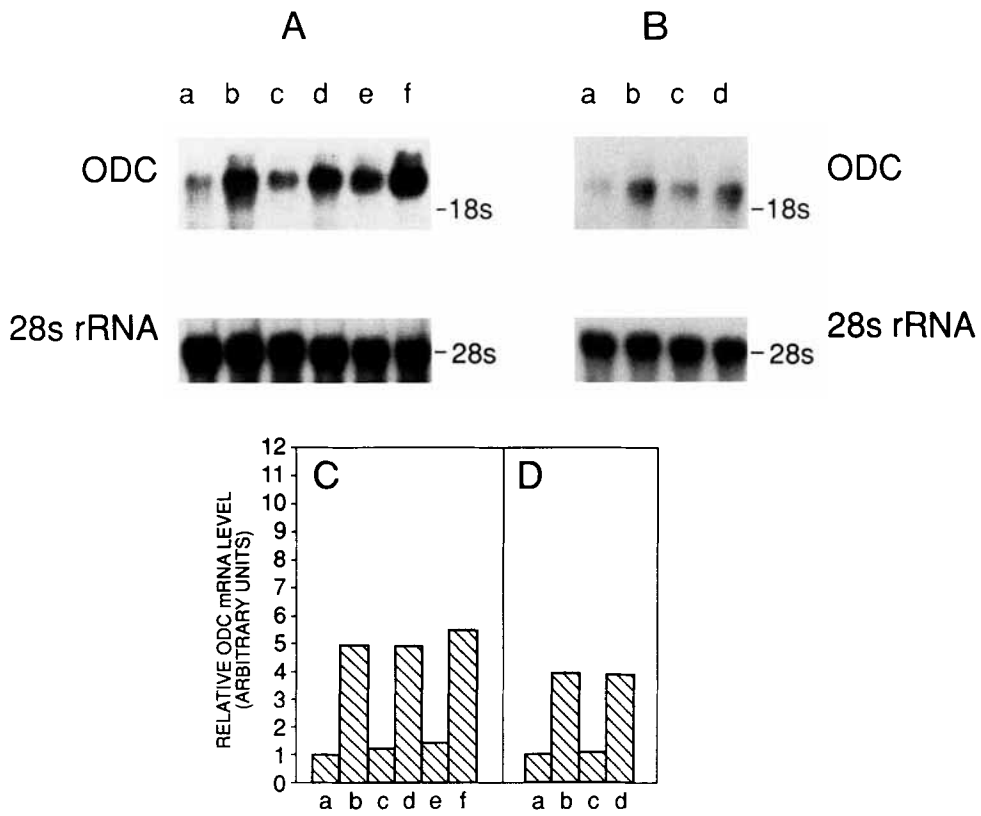


**Fig. 8.** **A:** Effect of protein kinase inhibitors on ODC mRNA levels in C2 cells treated with bFGF. Northern blot analyses are presented. C2 cells (control cells) (*lane a*); C2 cells exposed to bFGF (10 ng/ml) (*lane b*); H7, (50 μM) (*lane c*); H7 (50 μM) and bFGF (10 ng/ml) (*lane d*); HA1004 (50 μM) (*lane e*); HA1004 (50 μM) and bFGF (10 ng/ml) (*lane f*). Exposure time was 3 h. ODC and 28s rRNA autoradiograms were exposed at −70°C with intensifying screens for 24 h and 1 h, respectively. C2 cells were cultured in the absence of bFGF, H7, and HA1004 and exposed only to the vehicles within which these compounds were reconstituted. **B:** Effect of TPA pretreatment on ODC mRNA levels in C3 cells treated with bFGF. *Lane a:* RNA from untreated C3 cells. *Lane b:* RNA from C3 cells exposed to bFGF (10 ng/ml) for 1 h. *Lane c:* RNA from C3 cells pretreated with TPA for 48 h, no bFGF exposure. *Lane d:* RNA from C3 cells pretreated with TPA (0.1 μM) for 48 h, and then exposed to bFGF for 1 h. RNA levels from C3 cells shown in lanes a,b were nonpretreated C3 cells (no exposure to TPA). All autoradiograms were exposed at −70°C with intensifying screens. ODC and 28s rRNA autoradiograms shown were exposed for 6 h and 1 h, respectively. **C,D:** The densitometry data is shown for the autoradiographs provided in A and B, respectively.

observations obtained in TGF-β studies with these cells, where it was found that TGF-$\beta_1$ treatment modified both ribonucleotide reductase and ODC gene expression in malignant cells [Hurta et al., 1991, 1993]. This coordinate regulation is noteworthy, since ribonucleotide reductase and ornithine decarboxylase activities are critically linked to cell growth and survival processes [Wright, 1989; Pegg, 1988; Hayashi, 1989]. The observation that bFGF does not coordinately regulate these two gene activities as TGF-$\beta_1$ does suggests that these two growth factors may regulate ODC gene expression, but not ribonucleotide gene expression, via a shared signal transduction pathway.

Increased levels of ODC message were observed in malignant C3 cells following bFGF exposure at least partly due to modifications in the transcriptional efficiency of the ODC gene. However, changes at the posttranscriptional level in response to bFGF also occurred in malignant H-*ras* transformed cells, indicating that bFGF is capable of regulating ODC mRNA levels through a mechanism of posttranscriptional stabilization as well. The results of these studies suggest that the bFGF-mediated stabilization of ODC mRNA in C3 cells is part of an altered growth regulatory program associated with malignant transformation.

The cycloheximide-induced ODC gene expression observed in this study indicates that the ODC gene in malignant C3 cells can also be controlled in a positive manner by an inhibitor of protein synthesis. This behaviour is absent or

nonexpressed in normal 10T½ cells or tumorigenic but nonmetastatic NR3 cells [Hurta et al., 1993; Hurta and Wright, 1992]. This type of regulation may be caused by the presence of a labile protein repressor that disappears in the absence of protein synthesis or by the appearance of an activator which subsequently induces ODC gene transcription. The exact nature of this regulation of ODC expression in malignant cells remains to be determined. Interestingly, bFGF exposure in conjunction with protein synthesis inhibition by cycloheximide resulted in a further marked elevation of ODC mRNA expression. This result indicates the existence of a cycloheximide-sensitive regulator of ODC expression in the highly malignant H-*ras* transformed C3 cells.

The role of G-proteins in FGF-stimulated responses is controversial. Some studies have presented evidence to suggest that the stimulation of fibroblast and muscle cell proliferation by bFGF is entirely pertussis toxin–insensitive [Chambard et al., 1987; Kevin et al., 1989], whereas other investigations have suggested pertussis toxin inhibits growth of 3T3 cells [Logan and Logan, 1991]. Others have presented evidence to suggest that bFGF-stimulated endothelial cell movement is mediated by a pertussis toxin–sensitive pathway [Issandau and Darbon, 1991]. Regarding the role of possible pertussis toxin–sensitive G-proteins in the regulation of bFGF-mediated responses, our observations suggest that pertussis toxin–sensitive G-proteins may play a role in the bFGF-mediated alterations of ornithine decarboxylase gene expression in highly malignant H-*ras* transformed cells. The nature of the specific G-protein(s) and the signalling mechanism(s) involved are unknown and subject to further investigations.

Prolonged exposure of mammalian cells to TPA has been shown to result in a downregulation of PKC activity [Young et al., 1987]. TPA treatment of C3 cells causes a rapid but transient elevation of ODC gene expression with ODC mRNA levels returning to unstimulated levels following prolonged exposure to TPA (24–48 h). These results are consistent with previous observations obtained with BALBc/3T3 cells [Choy et al., 1989]. The observation that the bFGF-induced increase in ODC message levels in C3 cells is unaffected by TPA pretreatment (known to downregulate PKC activity) suggests that the bFGF modulation of ODC expression in C3 cells occurs independent of PKC-mediated events. This observation, coupled with the inability of PKC inhibitors to abrogate the bFGF-mediated elevation in ODC mRNA expression in malignant cells, suggests that the bFGF-mediated alterations in ODC mRNA expression in highly malignant H-*ras* transformed fibrosarcoma cells occurs through a protein kinase C–independent pathway. These observations are in keeping with several studies which suggest that the mitogenic/proliferative effects of FGFs may not be mediated by protein kinase C activation [Chambard et al., 1987; Issandau and Darbon, 1991; Kaibuchi et al., 1986; Magnaldo et al., 1986]. The exact nature of the signal transduction pathway involved in these malignant H-*ras* transformed cells remains to be further elucidated.

In summary, this study has demonstrated a novel relationship between bFGF signalling and ODC expression in H-*ras* transformed malignant fibrosarcomas. Further studies are required to determine how and what specific regulatory systems interact to control ODC in normal cells and how this regulation is abrogated or modified in highly malignant cells following bFGF exposure. The present report contributes to our understanding of the regulation of the malignant phenotype by bFGF and constitutes the basis for further investigations of the control mechanisms involved.

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## REFERENCES

Amara FM, Chen FY, Wright JA (1994): Phorbol ester modulation of a novel cytoplasmic binding activity at the 3′-untranslated region of mammalian ribonucleotide reductase R2 mRNA and role in message stability. J Biol Chem 269:6709–6715.

Amara FM, Chen FY, Wright JA (1995): Defining a novel *cis* element in the 3′-untranslated region of mammalian ribonucleotide reductase component R2 mRNA: Role in transforming growth factor-$\beta_1$ induced mRNA stabilization. Nucleic Acids Res 23:1461–1467.

Chambard JC, Pares S, L'Allemain G, Pouyssegur J (1987): Two growth factor signalling pathways in fibroblasts distinguished by pertussis toxin. Nature 326:800–803.

Chen FY, Amara FM, Wright JA (1993): Mammalian ribonucleotide reductase R1 mRNA stability under normal

and phorbol ester stimulating conditions: Involvement of a *cis-trans* interaction at the 3'-untranslated region. EMBO J 12:3977–3986.

Choy BK, McClarty GA, Wright JA (1989): Transient elevation of ribonucleotide reductase activity, M2 mRNA and M2 protein in BALB c/3T3 fibroblasts in the presence of 12-0-tetradecanoylphorbol-13-acetate. Biochem Biophys Res Commun 162:1417–1424.

Clifford A, Morgan D, Yuspa SH, Soler AP, Gilmour S (1995): Role of ornithine decarboxylase in epidermal tumorigenesis. Cancer Res 55:1680–1686.

Coughlin SR, Barr PJ, Cousen LS, Fretto LJ, Williams IT (1988): Acidic and basic fibroblast growth factors stimulate tyrosine kinase activity in vivo. J Biol Chem 263:988–993.

Cross M, Dexter TM (1991): Growth factors in development, transformation, and tumorigenesis. Cell 64:271–280.

Edwards DR, Parfett CLJ, Denhardt DT (1985): Transcriptional regulation of two serum induced RNAs in mouse fibroblasts: Equivalence of one species to B2 repetitive elements. Mol Cell Biol 5:3280–3288.

Egan SE, McClarty GA, Jarolim L, Wright JA, Spiro I, Hager G, Greenberg AH (1987): Expression of H-*ras* correlates with metastatic potential: Evidence of direct regulation of the metastatic phenotype in 10T½ and NIH 3T3 cells. Mol Cell Biol 7:830–837.

Fong WF, Heller JS, Canellakis ES (1976): The appearance of an ornithine decarboxylase inhibitory protein upon the addition of putrescine to cell cultures. Biochim Biophys Acta 428:456–465.

Gonzalez IL, Gorski JL, Campen TJ, Dorney DJ, Erickson JM, Sylvester JE, Schmeckel RD (1985): Variations among human 28S ribosomal RNA genes. Proc Natl Acad Sci USA 82:7666–7670.

Gough NM (1988): Rapid and quantitative preparation of cytoplasmic RNA from small numbers of cells. Anal Biochem 173:93–95.

Hayashi S (1989): "Ornithine Decarboxylase: Biology, Enzymology and Molecular Genetics." Elmsford, NY: Pergamon Press, Inc.

Heldin CH, Westermark B (1984): Growth factors: Mechanism of action and relation to oncogenes. Cell 37:9–20.

Hibshoosh H, Johnson M, Weinstein IB (1991): Effects of overexpression of ornithine decarboxylase (ODC) on growth control and oncogene-induced cell transformation. Oncogene 6:739–743.

Hidaka H, Inagaki M, Kawamoto S, Sasaki Y (1984): Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry 23:5036–5041.

Holtta E, Pohjanpelto P (1986): Control of ornithine decarboxylase in Chinese hamster ovary cells by polyamines. J Biol Chem 261:9502–9508.

Hovis JG, Stumpo DJ, Halsey DL, Blackshear PJ (1986): Effects of mitogens on ornithine decarboxylase activity and messenger RNA levels in normal and protein kinase C–deficient NIH-3T3 fibroblasts. J Biol Chem 261:10380–10386.

Huang A, Wright JA (1994): Fibroblast growth factor mediated alterations in drug resistance, and evidence of gene amplification. Oncogene 9:491–499.

Huang A, Jin H, Wright JA (1994): Aberrant expression of basic fibroblast growth factor in NIH-3T3 cells alters drug resistance and gene amplification potential. Exp Cell Res 213:335–339.

Hurta RAR, Wright JA (1992): Regulation of ribonucleotide reductase by the tumor promoters and protein phosphatase inhibitors okadaic acid, and calyculin A: Elevation of enzyme activity, R1 mRNA, R2 mRNA and R2 protein in BALBc/3T3 fibroblasts. Biochem Cell Biol 70:1081–1987.

Hurta RAR, Wright JA (1994): Alterations in the cyclic AMP signal transduction pathway regulating ribonucleotide reductase gene expression in malignant H-*ras* transformed cell lines. J Cell Physiol 158:187–197.

Hurta RAR, Wright JA (1995): Malignant transformation by H-*ras* results in aberrant regulation of ribonucleotide reductase gene expression by transforming growth factor-$\beta_1$. J Cell Biochem 57:543–556.

Hurta RAR, Samuel SK, Greenberg AH, Wright JA (1991): Early induction of ribonucleotide reductase gene expression by TGF-$\beta_1$ in malignant H-*ras* transformed cell lines. J Biol Chem 35:24097–24100.

Hurta RAR, Greenberg AH, Wright JA (1993): Transforming growth factor $\beta_1$ selectively regulates ornithine decarboxylase gene expression in malignant H-*ras* transformed fibrosarcoma cell lines. J Cell Physiol 156:272–279.

Issandau M, Darbon JM (1991): Basic fibroblast growth factor stimulates glomerular mesangial cell proliferation through a protein kinase C–independent pathway. Growth Factors 5:255–264.

Kahana C, Nathans D (1985): Translational regulation of mammalian ornithine decarboxylase by polyamines. J Biol Chem 260:15390–15393.

Kaibuchi K, Tsuda L, Kikuchi A, Tanimoto T, Yamashita T, Takai Y (1986): Possible involvement of protein kinase C and calcium ion in growth factor–induced expression of c-*myc* oncogene in Swiss 3T3 fibroblasts. J Biol Chem 261:1187–1192.

Katada T, Bokoch GM, Northup JK, Ui M, Gilman AG (1984): The inhibitory guanine nucleotide-binding regulatory compound of adenylate cyclase. Subunit dissociation and the inhibition of adenylate cyclase in S49 lymphoma cyc(−) and wild type membranes. J Biol Chem 259:3568–3577.

Katz A, Kahana C (1987): Transcriptional activation of mammalian ornithine decarboxylase during stimulated growth. Mol Cell Biol 7:2641–2643.

Kevin DJ, Simard G, Sue-A-Quan A, Connolly JA (1989): Growth factors, signaling pathways, and the regulation of proliferation and differentiation in BC3H1 muscle cells. II. Two signaling pathways distinguished by pertussis toxin and a potential role for the *ras* oncogene. J Cell Biol 108:169–176.

Kontula KK, Torkkeli TK, Bardin W, Janne DA (1984): Androgen induction of ornithine decarboxylase mRNA in mouse kidney as studied by complementary DNA. Proc Natl Acad Sci USA 81:731–735.

Le A, Ferrell GA, Dishon DS, Le QQ-A, Sifers RN (1992): Soluble aggregates of the human Pi-$Z_\alpha$-antitrypsin variant are degraded within the endoplasmic reticulum by a mechanism sensitive to inhibitors of protein synthesis. J Biol Chem 267:1072–1080.

Lee PL, Johnson DE, Cousen LS, Freid VA, Williams IT (1989): Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. Science 245:57–60.

Logan A, Logan SD (1991): Studies on the mechanisms of signalling and inhibition by pertussis toxin of fibroblast growth factor-stimulated mitogenesis in BALB/c 3T3 cells. Cell Signal 3:215–223.

Magnaldo I, L'Allemain G, Chambard JC, Moenner M, Barritault D, Pouyssegur J (1986): The mitogenic signaling pathway of fibroblast growth factor is not mediated through polyphosphoinositide hydrolysis and protein kinase C activation in hamster fibroblasts. J Biol Chem 261:16916–16922.

McClarty GA, Chan AKM, Wright JA (1986): Characterization of a mouse cell line selected for hydroxyurea resistance by a stepwise procedure: Drug dependent overproduction of ribonucleotide reductase. Somat Cell Mol Genet 12:121–131.

McClarty GA, Chan AK, Engstrom Y, Wright JA, Thelander L (1987): Elevated expression of M1 and M2 components and drug-induced post-transcriptional modulation of ribonucleotide reductase in a hydroxyurea resistant mouse cell line. Biochemistry 26:8004–8011.

McClarty GA, Chan AK, Choy BK, Wright JA (1990): Increased ferritin gene expression is associated with increased ribonucleotide reductase gene expression and the establishment of hydroxyurea resistance in mammalian cells. J Biol Chem 265:7539–7547.

Moshier JA, Dosescu J, Skunca M, Luk GD (1993): Transformation of NIH/3T3 cells by ornithine decarboxylase overexpression. Cancer Res 53:2618–2622.

Mukherjee R, Molloy GR (1987): 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole inhibits transcription of the beta-hemoglobin gene in vivo at initiation. J Biol Chem 262:13697–13705.

Olson EN, Spizz G (1986): Mitogens and protein synthesis inhibitors induce ornithine decarboxylase gene transcription through separate mechanisms in the $BC_2H1$ muscle cell line. Mol Cell Biol 6:2792–2799.

Pegg AE (1988): Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. Cancer Res. 48:759–774.

Pepper MS, Belin D, Montesano R, Orci L, Vassalli JD (1990): Transforming growth factor-beta 1 modulates basic fibroblast growth factor–induced proteolytic and angiogenic properties of endothelial cells in vitro. J Cell Biol 111:743–755.

Persson I, Oredsson SM, Anehus S, Heby O (1985): Ornithine decarboxylase inhibitors increase the cellular content of the enzyme: Implications for translational regulation. Biochem Biophys Res Commun 131:239–245.

Phillips DR, Crowthers DM (1986): Kinetics and sequence specificity of drug DNA interactions: An in vitro transcription assay. Biochemistry 25:7355–7362.

Presta M, Maier JAM, Ragnotti G (1989): The mitogenic signaling pathway but not the plasminogen activator-inducing pathway of basic fibroblast growth factor is mediated through protein kinase C in fetal bovine aortic endothelial cells. J Cell Biol 109:1877–1884.

Rifkin DB, Moscatelli D (1989): Recent developments in the cell biology of basic fibroblast growth factor. J Cell Biol 109:1–6.

Rizzino A, Ruff E, Rizzino H (1986): Induction and modulation of anchorage-independent growth by platelet-derived growth factor, fibroblast growth factor, and transforming growth factor-$\beta_1$. Cancer Res 46:2816–2820.

Ross J (in press): mRNA stability in mammalian cells. Microbiol Rev.

Russel DH, Snyder SH (1968): Amine synthesis in rapidly growing tissues: ornithine decarboxylase activity in regenerating rat liver, chick embryo, and various tumors. Proc Natl Acad Sci USA 60:1420–1427.

Schwarz LC, Gingras MC, Goldberg G, Greenberg AH, Wright JA (1988): Loss of growth factor dependence and conversion of transforming growth factor-$\beta_1$ inhibition to stimulation in metastatic H-*ras* transformed murine fibroblasts. Cancer Res 48:6999–7003.

Schwarz LC, Wright JA, Gingras MC, Kondaiah P, Danielpour D, Pimental M, Sporn MB, Greenberg AH (1990): Aberrant TGF-$\beta_1$ production and regulation in metastatic malignancy. Growth Factors 3:115–127.

Sertich GJ, Pegg AE (1987): Polyamine administration reduces ornithine decarboxylase activity without affecting its mRNA content. Biochem Biophys Res Commun 143: 424–430.

Sporn MB, Todaro GJ (1980): Autocrine secretion and malignant transformation of cells. N Engl J Med 303:878–880.

Tabor CW, Tabor H (1984): Polyamines. In Richardson CC, Abelson JN, Boyer PD, Meister A (eds): "Annual Review of Biochemistry," Vol. 53. Palo Alto, CA: Annual Reviews Inc., pp 749–790.

Wright JA (1989): Altered mammalian ribonucleoside diphosphate reductase from mutant cell lines. Int Encyclop Pharmacol Therapeut 128:89–111.

Wright JA, Egan SE, Greenberg AH (1990): Genetic regulation of metastatic progression. Anticancer Res 10:1247–1255.

Wright JA, Turley EA, Greenberg AH (1993): Transforming growth factor beta and fibroblast growth factor as promoters of tumor progression to malignancy. Crit Rev Oncog 4:473–492.

Yang L, Yang Y-C (1994): Regulation of interleukin (IL)-11 gene expression in IL-1 induced primate bone marrow stromal cells. J Biol Chem 269:32732–32739.

Yoshida M, Hayashi H, Taira M, Isonok (1992): Elevated expression of the ornithine decarboxylase gene in human esophageal cancer. Cancer Res 52:6671–6675.

Young S, Parker PJ, Ullrich A, Stabel S (1987): Down regulation of protein kinase C is due to an increased rate of degradation. Biochem J 244:775–779.