Neoplastic transformation of mouse mammary epithelial cells by deregulated *myc* expression

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A spontaneously immortalized, nontumorigenic mouse mammary epithelial cell line (MMEC) was transfected with an activated *myc* construct by electroporation. Constitutive expression of *myc* in MMEC resulted in anchorage independence in soft agar and tumorigenicity in nude mice. The *myc*-expressing MMEC showed higher saturation density, faster growth rate, and partial abrogation of serum-derived growth factor(s) requirement compared with parent MMEC. Epidermal growth factor or transforming growth factor alpha stimulated the anchorage-independent growth, but not the anchorage-dependent growth, of MMEC-*myc* cells. Type 1 transforming growth factor beta, on the other hand, inhibited both the anchorage-independent and anchorage-dependent growth of MMEC-*myc* cells. These results demonstrate that deregulated expression of *myc* results in neoplastic transformation in mammary epithelial cells. Accompanying the transformation is altered sensitivity to polypeptide growth factors.

Introduction

Activation of the *c-myc* proto-oncogene by gene rearrangements, amplification, and proviral insertions has been demonstrated in various malignancies (Battey *et al.*, 1983; Klein and Klein, 1985; Cory, 1986). *c-myc* amplification was found in a human breast carcinoma cell line (Kozbor and Croce, 1984) and in about one-third of primary breast cancer cells (Escot *et al.*,

1986). Mouse *c-myc* expressed from a mouse mammary tumor virus-long terminal repeat (MMTV-LTR) in transgenic mice predisposed the mice to mammary adenocarcinomas (Stewart *et al.*, 1984; Sinn *et al.*, 1987). Integration of the avian leukosis virus close to the *c-myc* gene in avian B-cell lymphomas resulted in enhanced *c-myc* transcription due to the proviral promoter/enhancer elements (Hayward *et al.*, 1981; Cullen *et al.*, 1984). Conceivably, deregulated expression of *c-myc* is the predominant mode of *myc* activation. The elevated levels of *c-myc* RNA that accompany the G0/G1 transition in some cell types after growth stimulation suggest that *c-myc* might play a role in the normal control of cell proliferation (Bister and Jansen, 1986). Thus, elevated cellular Myc expression may be associated with enhanced proliferation, a characteristic of transformed cells.

Transformation of mesenchymal or epithelial cells in vitro by oncogenic retroviruses results in partial or complete abrogation of the growth factor(s) requirement (Cherington *et al.*, 1979; Kaplan and Ozanne, 1982; Bradshaw and Dubes, 1983; Weissman and Aaronson, 1985). Oncogenes can also control the production and secretion of cellular growth factors, which function as distal effectors of cell transformation (Brown and Blakeley, 1984; Goustin *et al.*, 1986). Elevated synthesis and secretion of transforming growth factors (TGFs) has been implicated in the autocrine stimulation of various cell types (Brown and Blakeley, 1984; Goustin *et al.*, 1986). TGFs are polypeptides that were originally defined by their ability to induce anchorage-independent growth of nontransformed cells (De Larco and Todaro, 1978). Two types of TGFs, α and β , have been characterized. TGF α , a M_r 6000 polypeptide, has extensive sequence homology to epidermal growth factor (EGF) and is functionally related to it. TGF β , a powerful inhibitor of anchorage-dependent and -independent growth of both normal and transformed epithelial cells (Shipley *et al.*, 1986), cooperates with EGF (or TGF α) and platelet-derived growth factor (PDGF) to stimulate the anchorage-independent growth of NRK fibroblasts (Sporn and Roberts, 1985; Goustin *et al.*, 1986).

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Overexpression of *c-myc* in various cell types usually results in amplification of responsiveness to mitogenic growth factors (Stern *et al.*, 1986; Reiss *et al.*, 1989). BALB/c 3T3 cells transfected with *myc* show a partial abrogation of the PDGF requirement (Armelin *et al.*, 1984). Transfection of C3H/10T1/2 (murine fibroblasts) with an activated *c-myc* results in acquisition of responsiveness to TGF β -induced anchorage-independent growth (Leof *et al.*, 1987).

To understand the role of *c-myc* activation in mammary tumorigenesis, we have transfected activated *c-myc* into a nontumorigenic mouse mammary epithelial cell line (MMEC). Constitutive expression of *myc* in these cells resulted in neoplastic transformation. The *myc*-induced transformation of these cells was accompanied by altered sensitivity to growth factors compared with parent MMEC.

Results

Establishment of stable myc transfectants

MMEC, a nontumorigenic, contact-inhibited, anchorage-dependent cell line, was the recipient of DNA transfection. The mammary epithelial nature of MMEC was confirmed by the ability of these cells to repopulate the cleared (parenchyma-free) mammary fat pads. The transplantation sites showed the presence of well-formed ducts with prominent terminal end buds (Figure 1C).

We used electroporation as the mode of gene transfer into the MMEC. Because most epithelial cells are poorly transfectable by the conventional calcium phosphate technique (Redmond *et al.*, 1988), many investigators have used retrovirus-mediated gene transfer to transfect epithelial cells (Gunzberg *et al.*, 1988; Redmond *et al.*, 1988; Ball *et al.*, 1988). We have demonstrated high-efficiency, stable gene transfer by electroporation into a variety of cell types, including epidermal keratinocytes (Reiss *et al.*, 1986), bone marrow cells (Narayanan *et al.*, 1986), neuronal cells (Ito *et al.*, 1989), as well as many different primary cell types (Narayanan, unpublished observations). The feasibility of gene transfer into MMEC by electroporation was first established using a transient chloramphenicol acetyl transferase (CAT) assay (not shown). A recombinant *myc* construct, comprising the second and third coding exons of *myc* expressed from a Moloney murine leukemia virus-long terminal repeat (MLV-LTR) promoter in a NEO-derived vector (DM-*myc*, Lee *et al.*, 1985), was transfected into MMEC by electroporation, and stable G-418-resistant

clones were expanded in mass culture. Transfection efficiencies of 10^{-3} to 10^{-4} were routinely observed under our electroporation conditions. The *myc*-transfected MMEC was morphologically indistinguishable from the parent MMEC (Figure 1, A and B), but its morphogenetic pattern, as assayed by the mammary fat pad transplantation technique, was distinct. MMEC formed predominantly ductal outgrowths at the transplantation sites (Figure 1C), whereas MMEC-*myc*-3 cells formed highly hyperplastic lobuloalveolar outgrowths (Figure 1D). The constitutive expression of *myc* in these clones is demonstrated in Figure 2. The parent MMEC showed no detectable *c-myc* expression, whereas in the *myc*-transfected MMEC clones a 2.7-kb exogenous DM-*myc*-specific transcript was detected (Figure 2). Figure 3 shows that integrated DM-*myc*-specific sequences could be detected in the genomic DNA of several of these clones by Southern blot. Hybridization of *Sac* I digests of genomic DNA of MMEC-*myc* clones with a second exon-specific *c-myc* probe revealed the presence of a 2.8-kb exogenous DM-*myc*-specific band as predicted (Lee *et al.*, 1985; Reiss *et al.*, 1989). The 5.5- and 1.4-kb bands represent endogenous *c-myc* sequences. Two prototype clones, *myc*-1 and *myc*-3, were chosen for subsequent analysis.

Characterization of myc-transfected mammary epithelial cells

Although no gross morphological alterations were detected in the *myc*-transfected MMEC (Figure 1B), the MMEC-*myc* cells demonstrated a faster growth rate and a two- to threefold higher saturation density than the parent MMEC (Table 1). The MMEC-*myc* cells retained the contact-inhibited phenotype of the parent MMEC. Since the increased proliferation of MMEC-*myc* cells raised the possibility that they might have been transformed, we tested their ability to grow in soft agar. Interestingly, the MMEC-*myc* cells were anchorage independent (Table 1). The anchorage-independent growth of MMEC-*myc* cells suggested that they had been at least partially transformed; hence the tumorigenic potential of MMEC-*myc* cells was next investigated after subcutaneous injections into nude mice (Table 1). All the recipients showed rapidly growing tumors at the transplantation site within 3–5 wk after injection with 10^5 MMEC-*myc* cells. In contrast, none of the recipients transplanted subcutaneously with the parental MMEC showed tumors, even after 24 wk. Histopathological evaluation of the tumors

Figure 1. Morphology, transplantability, and tumorigenicity of MMEC and the MMEC-myc-3 cells. (A) Phase contrast photomicrograph of subconfluent culture of MMEC maintained in normal growth medium. Magnification, 50 $\times$. (B) Phase contrast photomicrograph of MMEC-myc-3 cells. Magnification, 50 $\times$. (C) Morphogenesis of transplanted MMEC in the cleared mammary fat pad. The cells have repopulated the transplantation site with ductal outgrowth showing the presence of ducts. The secondary ducts show prominent club-shaped terminal end buds. Magnification, 10 $\times$; hematoxylin. (D) Morphogenesis of MMEC-myc-3 transplanted into the cleared mammary fat pad. Intense hyperplastic growth is observed at the growing ends of the ducts. Magnification, 10 $\times$; hematoxylin. (E) Histopathology of the tumor arising in the mammary fat pad injected with MMEC-myc-3 cells. The presence of glandular elements, together with areas of undifferentiated cells, characterizes this adenocarcinoma. Magnification, 40 $\times$; hematoxylin-eosin.

Figure 2. Constitutive expression of *myc* in MMEC. Total RNA was analyzed by Northern blot hybridization with a ^{32}P -labeled *c-myc* second exon probe. Lane 1, RNA from MMEC; lanes 2–6, RNA from independent *myc* clones. Position of 28S and 18S is shown.

growing at the transplantation site confirmed them to be adenocarcinomas formed of glandular and nonglandular elements, suggesting heterogeneity of cytodifferentiation (data not shown). The MMEC-*myc*-3 cells were also tumorigenic when transplanted into cleared mammary fat pads of nude mice. The transplantation site showed highly proliferative hyperplastic outgrowths within a week (Figure 1D), and within 2–3 wk palpable tumors were noticed in the inguinal area. These tumors were adenocarcinomas (Figure 1E) similar to the tumors that resulted from subcutaneous injection. The tumors were reestablished in culture in the presence of G-418. The presence of DM-*myc*-related sequences was confirmed both in the tumors and tumor cell lines (Figure 3). No metastases were detected in the tumor-bearing animals.

Growth factor(s) requirement of *myc*-expressing MMEC under anchorage-dependent conditions

Many transformed cells demonstrate an ability to proliferate in low serum because of a reduced dependency upon exogenous serum-derived growth factors (Kaplan and Ozanne, 1983; Armelin *et al.*, 1984; Sporn and Roberts, 1985). Hence growth curves were constructed in reduced serum conditions (2% serum) to assay the growth factor requirements of the *myc*-transfected MMEC. In preliminary experiments with serum-free medium (0% serum) both the parent and the MMEC-*myc* cells died within 4–6 d. Figure 4 demonstrates the growth response of parent MMEC and MMEC-*myc*-1 cells to either EGF or TGF α under 2% serum conditions. At 2% serum, the parent MMEC ceased growth within 3 d, whereas the MMEC-*myc* cells continue to grow, although at a reduced rate compared with normal growth conditions. On the basis of a dose-response curve utilizing an anchorage-dependence assay, we chose a concentration of 5 ng/ml for both EGF and TGF α . Although both EGF and TGF α caused about a twofold stimulation in the growth of MMEC under reduced-serum conditions (Figure 4), neither of these growth factors caused significant stimulation of growth in MMEC-*myc*-1 cells under similar conditions. This demonstrates that deregulated *myc* in MMEC results in partial abrogation of the growth factor(s) requirement.

Growth response of MMEC-*myc* cells to EGF or TGF(s) in soft agar

MMEC-*myc* cells exhibited two properties associated with transformed cells: anchorage independence and tumorigenicity. Hence we investigated whether these transformed mam-

Table 1. Biologic characterization of *myc*-expressing MMEC

Cell line	Doubling time (h)	Saturation density $\times 10^6/\text{cm}^2$	Soft agar colony formation (%)	Nude mice tumorigenicity
MMEC	24.0	1.04 ± 0.2 (n = 3)	$<0.01 \pm 0.01$ (n = 4)	0/10 (24 wk)
MMEC- <i>myc</i> -1	18.2	2.29 ± 0.2 (n = 3)	3.8 ± 0.3 (n = 4)	10/10* (3–5 wk)
MMEC- <i>myc</i> -3	18.4	2.54 ± 0.3 (n = 3)	4.0 ± 0.6 (n = 4)	10/10* (3–5 wk)

Doubling time was determined from the linear portion of the growth curves. Saturation density was measured as the maximum number of cells obtained after initial plating with 4×10^3 cells/cm 2 and incubating with growth medium changed every 3 d. Cell suspensions (1×10^5 cells/ml) were plated in 0.33% soft agar in growth medium. Visible colonies were scored at days 10–12. Following injection with 10^5 cells, palpable tumors (*) were reestablished in tissue culture in the presence of G-418.

Figure 3. Detection of integrated *myc* sequences. Genomic DNA (20 μ g) from MMEC, MMEC-*myc* clones, one representative tumor tissue, and a cell line established from the same tumor, was digested with *Sac* I and analyzed by Southern blot hybridization with a 32 P-labeled *c-myc* second exon probe. Arrow indicates the position of exogenous DM-*myc*-specific fragment (Reiss *et al.*, 1989). DNA from lanes 1, *myc*-3 tumor tissue; lane 2, parent MMEC; lane 3, *myc*-1; lane 4, *myc*-3; lane 5, *myc*-4; and lane 6, *myc*-3 tumor cell line (G-418). Lanes 3 and 5 contain a lower amount of DNA (10 μ g).

mary epithelial cells might differ from the parent MMEC in their anchorage-independent growth responsiveness to EGF, TGF α , or TGF β . Cells were grown in soft agar in the presence or absence of EGF (5 ng/ml), TGF α (5 ng/ml), or TGF β 1 (3 ng/ml) for 10–12 d. As shown in Figure 5, neither EGF nor TGF α stimulated anchorage-independent growth in the parent MMEC. Occasionally, small clusters (<10 μ m) were observed that did not grow. However, both EGF and TGF α caused an approximate two- to threefold stimulation in colony size and numbers of the *myc* transfectants. TGF β , which has both

growth stimulatory and inhibitory effects (Roberts *et al.*, 1980; Anzano *et al.*, 1982; Tucker *et al.*, 1983; Roberts *et al.*, 1985; Moses *et al.*, 1985; Salomon *et al.*, 1987), requires either EGF or TGF α to stimulate the anchorage-independent growth of untransformed NRK cells (Sporn and Roberts, 1985; Roberts *et al.*, 1985; Goustin *et al.*, 1986). In a mouse mammary epithelial cell line, TGF β was shown to enhance synergistically the effects of EGF under anchorage-independent conditions (Salomon *et al.*, 1987). Hence we investigated the effects of TGF β on the anchorage-independent growth of MMEC-*myc* cells. TGF β 1 did not induce anchorage-independent growth in MMEC. Furthermore, TGF β 1 caused a threefold reduction in the number of colonies formed in the MMEC-*myc*-1 clone (Figure 5). The inhibition of anchorage independence in the *myc*-1 clone by TGF β 1 was not relieved by the addition of optimum concentrations of TGF α . In addition, the inhibition of MMEC-*myc* cells' proliferation by TGF β 1 was observed even under anchorage-dependent conditions (Figure 6).

Discussion

The mechanism by which *myc* transforms cells is far from clear. *myc* has been shown to bind to DNA (Donner *et al.*, 1982), is located in the nucleus (Abrams *et al.*, 1982; Eisenman *et al.*, 1985), and is associated with the nuclear matrix in vivo (Klein, 1983), but the function of *myc* is yet to be identified. To understand the role of *myc* in cellular transformation, several groups have transfected *v-myc* and *c-myc* genes into rodent fibroblasts and epithelial cells, either by viral infection or DNA-mediated transfection. Transformation of mammalian cells by the MC29 virus is not efficient (Copeland *et al.*, 1980). However, using an experimentally derived murine retrovirus that encodes avian *v-myc*, it is possible to transform murine fibroblasts and macrophages (Vennstrom *et al.*, 1984). *v-myc*-transfected bladder epithelial cells show partial transformation in vitro by being able to grow in soft agar (Falcone *et al.*, 1987). A second gene such as *ras*, polyoma large T antigen, or adenovirus early region gene 1A is frequently necessary (Keath *et al.*, 1984; Lee *et al.*, 1985; Salomon *et al.*, 1987) to transform cells by deregulated *myc*. Keath *et al.*, (1984) have demonstrated complete transformation of NIH-3T3 and RAT-2 fibroblasts with an activated mouse *c-myc* gene, similar to our current findings with an activated human *c-myc* gene. The cell line used in our studies, MMEC, did not

Figure 4. Serum growth factor requirement of MMEC-myc-1 transfectants. Cells (5×10^3) were plated in duplicate wells (6 wells, Costar) in growth medium. After 24 h, the medium was removed, cells were washed with PBS and were incubated in low serum medium (2% FBS) for 24 h. EGF (5 ng/ml) or TGF α (5 ng/ml) was added and the cells were incubated for 3 d with one medium change. Viable cells (Trypan blue) were counted. Results are average values from duplicate wells in a representative experiment. (—), no addition.

Figure 5. Effect of EGF and TGF on the anchorage-independent growth of MMEC-myc-1 cells. Cells (1×10^3 /well) in duplicate were plated in 0.3% agar containing growth medium $\pm$ EGF (5 ng/ml), TGF α (5 ng/ml), TGF β 1 (3 ng/ml), or TGF α (5 ng/ml) plus TGF β 1 (3 ng/ml). Colonies were counted at day 10. The growth factor concentrations used were those found to give optimum response.

Figure 6. Growth inhibition of MMEC-myc-1 clone by TGF β under anchorage-dependent conditions. Cells (5×10^3 /well) in duplicate were plated in growth medium. After 48 h, cells were incubated with or without TGF β 1 (3 ng/ml) for 4 d with one medium change. On day 4, cells were trypsinized and viable cells were counted. Results are averages from a representative experiment.

show any activation of *ras* P-21 (not shown). Thus the neoplastic transformation of MMEC by *myc* is probably not due to a cooperative interaction with a preexisting *ras* mutation. However, the possibility exists that some other putative oncogenes are activated in these cells.

Hormone-inducible expression of the casein gene, a marker for differentiation in mammary epithelial cells (Danielson *et al.*, 1984), was not detected in the MMEC, suggesting a block in the differentiation pathway. The de-differentiated status of the MMEC might also have contributed to the ease with which these cells were transformed by deregulated *myc*. Efforts are currently underway to transfect various MMEC lines with the same *myc* construct.

An immortalized human mammary epithelial cell line, 184A1 (Clark *et al.*, 1988), showed similar alterations in its differentiated status. An MMEC line, HC-14 (Ball *et al.*, 1988), has been shown to express very low levels of β -casein milk protein despite its ability to repopulate the cleared mammary fat pads. However, the mammary epithelial nature of the MMEC line used in the current study can be inferred by our observation that transplanted MMEC retain the ability to repopulate the transplantation site with mammary ducts, as well as by the MMEC-*myc*-3 cells' hyperplastic growth and formation of adenocarcinomas at the transplantation sites.

Our present observations from the transplantation experiments essentially confirm and extend the previous findings on murine mammary epithelium in vitro (Telang *et al.*, 1979; Iyer and Banerjee, 1981; Guzman *et al.*, 1987; Miyamoto *et al.*, 1988). The growth stimulation of the parental MMEC but not the MMEC-*myc* cells under anchorage-dependent conditions by EGF or TGF α implies that constitutive Myc expression had caused an abrogation of the requirement for these growth factors. However, the effects of EGF or TGF α upon the growth of MMEC-*myc* cells were significantly different when monitored under anchorage-independent conditions. Both EGF and TGF α stimulated the soft agar colony formation of MMEC-*myc* cells, whereas the parental MMEC was unresponsive.

It is not surprising that the parental MMEC was unresponsive to the mitogenic effects of EGF or TGF α in the anchorage independence assay yet sensitive to stimulation of anchorage-dependent growth. In primary cultures of rat or mouse mammary epithelial cells it has been demonstrated that EGF or TGF α is not effective in stimulating growth on soft agar, although both of these growth factors are mitogenic for these cells in monolayer culture (Zweibel *et al.*, 1982). Similarly, it was recently shown that *ras*-transfected mouse mammary epithelial cells were refractory to the growth-promoting effects

of exogenous EGF or TGF α , whereas the parental cells were sensitive (Salomon *et al.*, 1987). MMEC is a spontaneously immortalized cell line, but immortalization has not sensitized the cells to the mitogenic activity of EGF or TGF α under anchorage-independent conditions, since the parent cells did not form colonies, even in response to these growth factors.

Recently it has been reported that TGF β causes downregulation of *c-myc* in a murine keratinocyte cell line (Coffey *et al.*, 1987; Pietenpol *et al.*, 1990). These authors have suggested that growth inhibition of these cells by TGF β might be mediated through inhibition of *c-myc* expression. If downregulation of *c-myc* were the predominant mode of action of TGF β in inhibition of cell growth, the presence of deregulated *myc* expression in cells would be expected to result in partial or complete abrogation of the growth-inhibitory action of TGF β . This has not been the case in either of two distinct epithelial cell systems. In the same murine keratinocyte cell line (BALB-MK) Pietenpol *et al.* (1990) have used, TGF β inhibited EGF-induced proliferation in the presence of constitutive *myc* expression, as we have shown (Reiss *et al.*, 1989). In this study, using a totally different epithelial cell line, we further demonstrate that deregulated *myc* does not alter the growth-inhibiting activity of TGF β . Thus, even in the presence of persistent *myc* expression, the antiproliferative activity of TGF β is not diminished, which implies that the inhibitory action of TGF β in epithelial cell systems may involve regulatory pathways other than *myc*. TGF β 's mechanism of action is further complicated by its bifunctionality: growth inhibition and growth stimulation. Clearly, TGF β 's mechanism of action is little understood. It is likely that inhibition of cell growth by TGF β involves many different pathways.

In summary, we demonstrate that deregulated expression of *myc* in a mouse mammary epithelial cell line (MMEC) results in neoplastic transformation. The *myc*-transformed MMEC showed significantly altered growth properties and changes in sensitivity to polypeptide growth factors in comparison to the untransformed MMEC.

Materials and methods

Establishment and transfection of MMEC cell line

The mouse mammary epithelial cell line MMEC was established from 8-wk-old virgin female BALB/c mice. The inguinal (#4) mammary glands from eight animals were pooled and

finely minced with scalpels. The tissue mince was enzymatically digested in a 1:1 mixture of 0.01% collagenase (type IV-S, Sigma, St. Louis, MO) and 0.01% hyaluronidase (Sigma) by incubation in a shaker water bath at 37°C for 90 minutes. The tissue digest was then centrifuged at 1000 r.p.m. at room temperature for 5 min, and the pellet was resuspended for culturing in Dulbecco's modified Eagle's medium (DMEM). The additives to the basal medium included 2 mM L-glutamine, 5 μ g/ml porcine insulin (Eli Lilly, Indianapolis, IN), and 10% fetal bovine serum. Routinely, the culture medium contained 100 U/ml penicillin, 100 μ g/ml streptomycin, 100 μ g/ml gentamycin, and 4 μ g/ml amphotericin B as antibiotics. The primary cultures were plated in 100 mm plastic petri plates (Falcon Plastics, Becton/Dickinson, Rahway, NJ) and were maintained in an atmosphere of 95% air:5% CO₂ at 37°C. Initially, the cultures were left undisturbed for at least 5–7 days. By this time epithelioid colonies were visible. These epithelioid colonies were then trypsinized using cloning cylinders and mass cultured. MMEC was routinely passaged (1:10 split) at 70–80% confluence. The cell line at p-30 was used for transfection.

MMEC was grown in plastic dishes in DMEM with additives as detailed above. Supercoiled DM-*myc* plasmid DNA (Lee *et al.*, 1985) was transfected into MMEC by electroporation (Narayanan *et al.*, 1986; Reiss *et al.*, 1986; Ito *et al.*, 1989). Individual G-418-resistant clones (400 μ g/ml) were picked with cloning cylinders and maintained in G-418-containing medium. Nucleic acids were analyzed by hybridization using ³²P-labeled nick-translated probes as described (Reiss *et al.*, 1989; Ito *et al.*, 1990). A 1.8-kb *Sac* I fragment of human *c-myc* spanning the second exon was used as probe.

Growth factor dependence assay

Suspensions of single cells at a density of 1×10^4 cells were seeded in 6 cm² Costar (Cambridge, MA) plates in normal medium. After an initial attachment of 24–48 h, various growth factors were added to the medium as shown. EGF was from Collaborative Research, Inc., Waltham, MA. TGF α and TGF β 1 were from R & D Systems, Minneapolis, MN. Duplicate cultures were trypsinized and counted by hemocytometer at indicated times. Each experiment was performed three times, and all the growth factor experiments were done with two independent MMEC-*myc* clones. Since both of the clones showed the same effects only one, *myc*-1, is shown.

Anchorage independence assay

Cells (1×10^3) from a single cell suspension were plated in 12-well Costar dishes in 1 ml of 0.3% Difco Noble agar, which was supplemented with complete growth medium. The effects of growth factors on the anchorage-independent growth of the MMEC and MMEC-*myc* cells were tested by adding various growth factors at the indicated concentrations to the cell suspension prior to seeding. The dishes were subsequently incubated at 37°C for 10–12 d. Macroscopic colonies (>40 μ m) were counted from duplicate wells. Each anchorage independence assay was performed 3 times.

Tumorigenicity

The tumorigenicity of the mouse mammary epithelial cells was analyzed by injecting 10^5 cells subcutaneously into 6- to 8-wk-old female nude mice (BALB/c). The transplantation sites were palpated twice weekly and tumors were allowed to grow to at least 1.5–2.0 cm before sacrifice. Tumors were

surgically excised for histological examination. The sacrificed animals were examined for metastases. Tumors were re-established in culture in the presence of G-418.

Mammary fat pad transplantation

The technique of mammary fat pad transplantation used was essentially similar to that reported previously (Telang *et al.*, 1979; Iyer and Banerjee, 1981). Briefly, 10^5 cells in a 20 μ l suspension were injected as a bolus into the inguinal (#4) mammary fat pads of nude mice. The recipient mice were maintained until the tumors attained a mean diameter of 1.0–1.5 cm. The recipients were sacrificed, inguinal fat pads were excised in their entirety, and were processed for histopathological examination as described (Telang *et al.*, 1979; Telang and Sarkar, 1983).

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