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An *in vitro* model of epithelial cell growth stimulation in the rodent mammary gland

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Abstract. Mouse mammary epithelial cell cultures previously described bring about extensive proliferation and a cell population with the appropriate markers for luminal ductal epithelial cells, and also the ability to form normal tissue after implantation into mice. This success may result from a culture environment that resembles certain aspects of the environment in the mammary gland. Mouse mammary epithelial cells, whose proliferation is limited when plated alone, can be stimulated to multiply by contact with lethally irradiated cells of the LA7 rat mammary tumour line. Most of the proliferative stimulus is imparted by direct cell contact between LA7 and mouse mammary cells. Junctions, including adherens junctions, form among all cells in the culture, much as junctions form in the mammary gland. LA7 cells secrete TGFα and bFGF, factors found in the mammary gland, and factors to which mouse mammary cells respond in culture. Mouse mammary cells express keratins 8 and 18, markers for luminal cells of the mammary duct. LA7 cells express keratin 14 and vimentin, markers for myoepithelial cells. These facts, taken together, fit a model of cell replacement in an epithelial tissue and also imitate the relationship between luminal ductal cells and myoepithelial cells in the mammary gland. This method of culturing cells is useful, not only for *in vitro–in vivo* carcinogenesis studies, but also for the study of mechanisms by which growth signals are imparted from one cell to another.

INTRODUCTION

Historically, epithelial cells, compared with other cell types, have been difficult to culture. Now, even with published methods for various epithelial cells, the ones which promote a number of cell doublings approaching the expected limit, which result in a cell population with markers of the desired cell type, or which successfully function in animals or humans after implantation, is small. In this respect, our previously published unique method for culturing mouse mammary

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epithelial cells (MMEC) has been particularly successful. One reason for this success may be the similarity of the culture system to the *in vivo* condition of the cells. Here, we present findings that demonstrate some of these similarities.

Normal MMEC, taken directly from tissue, can multiply to 10–20 times the original cell number when plated by themselves in primary culture (Imagawa *et al*. 1982), but have resisted substantial proliferation after subculture. However, growth stimulation can be achieved if the MMEC are cultured in direct contact with lethally irradiated cells of the immortal LA7 rat mammary tumour line. By this method, MMEC can be cultured for at least 12 passages and 30 doublings in cell number (Ehmann *et al*. 1984), and their ultimate life span capacity in culture is not known. The cultured cells form normal-appearing glandular structures when implanted into mammary fat pads (Ehmann *et al*. 1987). Here, we present evidence that they also retain markers of normal mammary epithelial cells.

This unusual culture method was conceived to be an *in vitro* model of cell replacement in epithelial tissue. Epithelial cells, unlike other cell types such as fibroblasts, are always connected *in vivo* by direct contact via tight junctions that form an intact barrier to specific ions, molecules, and solutes. The integrity of this barrier must be maintained, even when a cell within the tissue dies. Therefore, some signal(s) for the healthy cells to divide must be transmitted when a damaged cell is lost from the confluent epithelial tissue. Cells of the LA7 rat mammary line were chosen as surrogates that the MMEC might recognize as being similar and with which they might form a demi-epithelial tissue *in vitro* if the two cell types were plated together at a confluent density. Indeed, as the lethally irradiated LA7 cells slowly die, the MMEC divide to occupy the spaces of the dying LA7 cells. In this way, the confluent monolayer slowly changes from one composed mostly of irradiated LA7 cells to one composed entirely of MMEC (Ehmann *et al*. 1984). This suggests that the culture system is a model for replacement of wounded epithelial cells.

The propagation of MMEC probably depends on both production of growth factors by the LA7 cells and on formation of physical connections between LA7 cells and MMEC. We now present data indicating that the LA7 cells produce at least two growth factors known to operate in the mammary gland, namely, transforming growth factor α (TGFα) and basic fibroblast growth factor (bFGF). Close juxtaposition of the two cell types provides the opportunity for stimulation by membrane-bound growth factors, and confluency of the mixed MMEC-LA7 culture is necessary for maximum proliferation of MMEC (Ehmann 1992). LA7 cells also form junctional complexes with MMEC, including tight junctions and desmosomes (Ehmann *et al*. 1998). The junctional complexes and growth factors involved here are those found in the intact mammary gland.

The usefulness of this culture system is two-fold. First, the ability to transfer MMEC from culture into the animal facilitates studies of proliferation, carcinogenesis and cell aging. Secondly, that the *in vitro* environment nurturing MMEC proliferation mimics the *in vivo* situation allows extrapolation of conclusions reached about cell–cell interactions in culture to cell–cell interactions in the mammary gland.

MATERIALS AND METHODS

Mouse mammary epithelial cell culture

The culture of MMEC has been previously described (Ehmann *et al*. 1984). MMEC cultures were prepared from glands excised aseptically from 6- to 8-week-old female Balb/c mice (Simonson Laboratories, Gilroy, CA, USA), minced, and digested overnight with 0.1% collagenase

(Worthington Biochemicals, Malvern, PA, USA) in culture medium (see below) at 37 °C. The tissue suspension was poured through a 65-μm nytex filter to capture the epithelial organoids, but allow most single cells (including fibroblasts) to pass through. The organoids were introduced into tissue culture flasks whose growth surface was covered with lethally irradiated LA7 cells. The cells were fed with fresh medium three times per week until the growth surface was covered with a confluent monolayer of the normal epithelial cells, usually ~5 weeks. The cultures were maintained in a 1 : 1 combination of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (Hazleton Biologics, Lenexa, KS, USA) supplemented with 10 μg/ml insulin, 5 μg/ml transferrin, 0.2 mM (120 U/ml) penicillin G, 0.05 mM streptomycin sulphate (Sigma, St Louis, MO, USA), 20 mM HEPES buffer, 2.0 g/l $NaHCO_3$, and 0–10% fetal bovine serum (Sterile Systems, Logan, UT, USA) or new-born calf serum (Sigma), and a supplement containing the trace elements Se, Zn, Cu, V, Si, Mn, Sn, and Ni (Hammond *et al*. 1984).

MMEC subcultured to passages 3–5 from primary cultures were used in most experiments. To prepare subcultures, the cells were dislodged from the plastic by incubation in a trypsin-EDTA phosphate-buffered saline (PBS) solution. The cells were introduced into a new flask at the desired dilution, usually 1 : 5–1 : 20, from which they again proliferated to cover the flask surface in about 5 weeks if fresh irradiated LA7 cells were provided.

LA7 cells

Rat mammary cells of the LA7 line were originally obtained from Dr Renato Dulbecco (The Salk Institute, San Diego, CA, USA). These cells are a clonal derivative of the Rama 25 line, which came from a Sprague-Dawley rat treated with dimethylbenz(a)-anthracene (Dulbecco *et al*. 1979). Before plating the normal epithelial cells, the LA7 cells were proliferatively inactivated by a single dose of 60 Gy (6000 rads) from a ^{137}Cs source. Culture medium for the LA7 cells alone consisted of 1 : 1 DMEM/F12 base supplemented with 10 μg/ml insulin, 50 nM hydrocortisone, 0.2 mM penicillin G, 0.05 mM streptomycin sulphate, and 5% new-born calf serum. MDA-MB-231 cells, from a breast adenocarcinoma, used as a positive control known to secrete TGFα, were cultured and treated the same as the LA7 cells.

Conditioned media

LA7 or MDA-MB-231 cells were diluted into tissue culture flasks in passages 30–35 and fed three times per week with culture medium as described above. When the cells became nearly confluent, the medium was changed to include only 1.0 μg/ml insulin, and the trace elements mentioned above without any serum. At each feeding, the medium was collected and frozen and replaced with fresh medium. After several collections, the proteins in the pooled media were concentrated by a factor of 30 in an Amicon 600-ml thin channel system fitted with a 3000 molecular weight cut-off filter. This concentrate was sterilized by passage through a 0.20-μ filter and mixed with fresh medium to achieve a final concentration of proteins of 20 times that in the original conditioned media.

MMEC growth curves

MMEC were removed from a confluent secondary culture by trypsin/EDTA and plated in medium with 10% new-born calf serum plus insulin, transferrin, antibiotics and trace elements. About 5×10^4 cells/35-mm dish were plated in experiments to which the growth factors TGFα and bFGF were added, and ~10^5 cells/35-mm dish were plated in experiments to study the effects of irradiated LA7 cells or conditioned medium from them. The next day, the dishes were divided into experimental groups, and growth factors, conditioned media, or feeder cells, as appropriate, were added along with the appropriate growth factors and 0.5% serum plus all other supplements. All cultures were fed three times per week with the appropriate media, and a dish

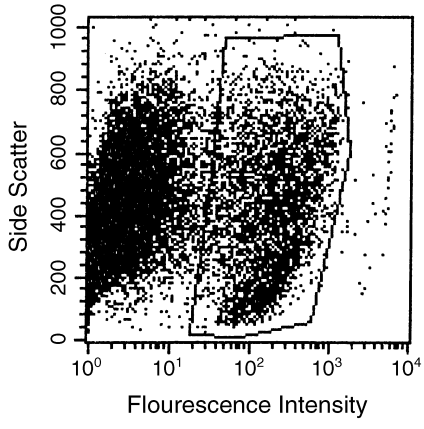

Figure 1. FACScan analysis of percentages of MMEC in co-culture with LA7 feeder cells on day 7 after plating. Polygon defines population of MMEC, i.e. those that reacted with the anti-MMEC serum. Twenty-two per cent of the cell population were MMEC.

from each group was removed every 3–4 days for analysis. The cells for analysis were trypsinized and a portion counted on a Coulter Counter.

In co-cultures the remainder were analysed for mouse/rat, i.e. MMEC/LA7, cell ratio (Fig. 1). The sample was incubated with an anti-mouse serum raised in Sprague-Dawley rats injected with dead MMEC (Josman Laboratories, Napa, CA). Subsequently, the sample was incubated with an FITC-conjugated rabbit anti-rat IgG serum. After fixation with 1% para-formaldehyde, the fraction of fluorescently labelled mouse cells was determined by analysis on a Becton Dickenson FACScan (Fig. 1). To obtain the number of epithelial cells in the sample, the fraction of fluorescently labelled cells was multiplied by the total cell count.

Immunocytochemistry

LA7 cells were plated into T25 tissue culture flasks in passage 54 and fed for 1–2 days until they were confluent. After irradiation of the LA7 cells, 1000 MMEC were seeded onto the monolayer. After regular feeding, MMEC colonies appeared after about 6 weeks. Before fixation and between every step of immunocytochemical staining, the cells were washed thoroughly with PBS. The cells were fixed with methanol (10 min, −80 °C), and then permeabilized with 0.5% triton-X-100 in PBS. The first stage antisera (Table 1) were applied to the monolayers and incubated at 37 °C for 1.5 h, followed by 1 h. incubation with the second stage sera (Table 1). The cells were viewed by epifluorescence on a Nikon microscope and photographed with a Nikon 35-mm N2000 camera and 400 ASA black-and-white film.

Immunoblotting

The 30 × conditioned medium described above was further concentrated to 230 ×. Proteins in the concentrated medium were separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli 1970). The samples prepared for detection of TGFα, EGF, and bFGF were separated on a tricine gel consisting of 16.5% T and 3% C resolving gel, 10% T and 3% C spacer gel, and 4% T and 3% C stacking gel, where T denotes total percentage concentration of acrylamide and bisacrylamide and C denotes percentage concentration of the crosslinker relative to total concentration of T (Schagger & von Jagow 1987). The proteins were transferred from the gels to a nitrocellulose membrane, and then probed with mouse monoclonal

Table 1. Antibodies for immunocytochemistry

Antigen	First antibody		Vendor or reference	Second antibody[b]		
	Source[a]	Dilution		Source	Dilution	Vendor
TGFα	Rabbit p	1 : 100	Research & Diagnostic Ab	Goat	1 : 160	Sigma
bFGF	Rabbit p	1 : 50	Sigma	Goat	1 : 160	Sigma
Keratin 8	Rat m	1 : 40	DSHB[c]	Rabbit	1 : 20	Vector
Keratin 14	Mouse m	1 : 20	Neomarkers	Horse	1 : 100	Vector
Keratin 18	Rabbit p	1 : 25	Ku, *et al.* (1995)	Goat	1 : 100	Sigma
Vimentin	Mouse m	1 : 30	Sigma	Horse	1 : 100	Vector
E-cadherin	Rat m	1 : 250	Sigma	Rabbit	1 : 20	Vector
Epithelial membrane antigen	Mouse m	0	Dako	Primary FITC-conjugated		

[a]m, monoclonal; p, polyclonal. [b]All second-stage antibodies were FITC-conjugated. [c]Developmental Studies Hybridoma Bank, University of Iowa.

anti-TGFα (2 μg/ml), anti-bFGF (1 : 1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or rabbit anti-TGFα (1 : 1000), or anti-bFGF (1 : 1500) (both from Sigma). The reacted bands were visualized with enhanced chemiluminescence.

RESULTS

MMEC did not proliferate noticeably when plated onto tissue culture plastic alone, even in medium containing insulin and transferrin (Fig. 2). However, if they were surrounded by a confluent monolayer of irradiated cells of the LA7 rat mammary tumour line, they multiplied and gradually replaced the dying LA7 cells (Figs 1 and 2). LA7 rat mammary tumour cells produced factors that stimulated MMEC proliferation because MMEC numbers increased when medium conditioned by LA7 cells was concentrated and applied to MMEC cultures (Fig. 2). The resulting increase was not as great as when MMEC were co-cultured with the LA7 cells themselves, but was greater than that of MMEC cultured in unconditioned medium, where the cell number decreased with time (Fig. 2).

We have identified two growth factors produced by LA7 cells, namely, TGFα and bFGF. Antisera to both TGFα and bFGF labelled cultured, fixed LA7 cells, whether they were unirradiated (Fig. 3) or irradiated (not shown). The antiserum to TGFα also labelled cultured, fixed MDA-MB-231 breast carcinoma cells, which are known to secrete TGFα (not shown) (Dickson *et al*. 1986). To determine whether the growth factors were secreted into the medium, proteins from concentrated LA7 conditioned media were separated by SDS–PAGE and probed with antisera. Both TGFα and bFGF proteins were detected on the blots from these separations (Fig. 4). A major band reacted with anti-TGFα serum and co-migrated with synthetic TGFα (5.6 kDa), and several other higher molecular weight bands, a wide one of which was 55 kDa, were also visible (Fig. 4). A weak band reacted to anti-bFGF serum at 12 kDa, the same molecular weight as that of the minor band of synthetic bFGF, and a heavy band was visible at ~31 kDa.

Both TGFα and bFGF elicited proliferative responses in MMEC plated alone and fed with media to which each growth factor had been added (Fig. 5). Addition of both growth factors together elicited a greater response than either one alone. Although the increase in cell number was small and unsustained, the cell numbers in those cultures remained higher than in MMEC

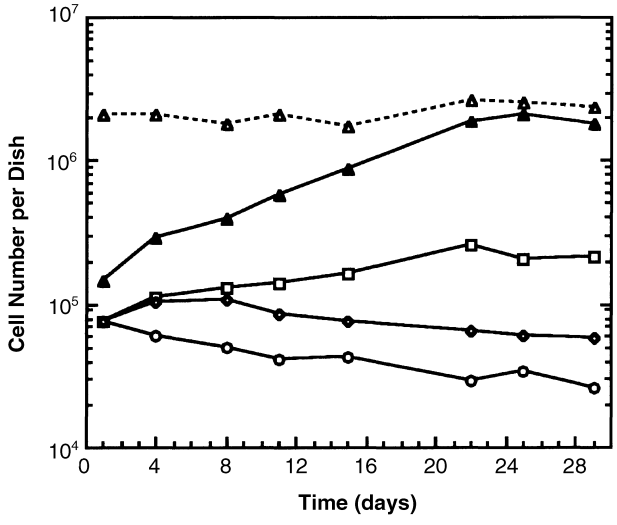

Figure 2. Proliferation of MMEC with conditioned media or LA7 feeder cells. (○) MMEC alone in usual culture medium; (◇) MMEC alone in medium conditioned by MDA-MB-231 cells, which secrete TGFα; (□) MMEC alone in medium conditioned by LA7 cells; (▲) MMEC co-cultured with lethally irradiated LA7 cells; (Δ) total number of MMEC + LA7 feeders in co-culture. All data points were obtained by FACs analysis similar to that of Figure 1. This experiment is representative of all experiments of this type. Each curve on the graph differs significantly from each other curve at $P < 0.01$.

cultures fed with control medium. The proliferative response brought about by synthetic TGFα was mirrored by increased MMEC proliferation in medium conditioned by TGFα-secreting MDA-MB-231 cells (Fig. 2).

The physical connections between MMEC and LA7 feeders were explored in a previous communication (Ehmann *et al*. 1998). At that time, we were not able to detect adherens junctions by immunocytochemistry even though β-catenin, which normally binds cadherins, was visible, and E-cadherin was present in LA7 cell lysates. In more recent experiments, E-cadherin between some LA7 cells and adjacent MMEC was detectable by immunocytochemistry (Fig. 6). These appeared dimmer than junctions among MMEC themselves. That they have not always been visible is probably related to the hybrid nature of the adherens junctions formed between the two cell types and the specificity of the antibody.

Even though both the MMEC and LA7 cells originated in rodent mammary glands, they expressed antigens characteristic of different cell types. The MMEC strongly expressed markers of luminal ductal epithelial cells, namely, keratins 8 (Fig. 7) and 18, and they moderately expressed epithelial membrane antigen. LA7 cells, on the other hand, did not express those keratins, but, instead, expressed keratin 14 (Ehmann & Terris 2002) and vimentin (Fig. 8), both of which are characteristic of myoepithelial cells.

DISCUSSION

Many elements of the *in vivo* environment from which MMEC derive are present in the culture system described here. The growth factors, TGFα and bFGF, which stimulate mammary cells *in*

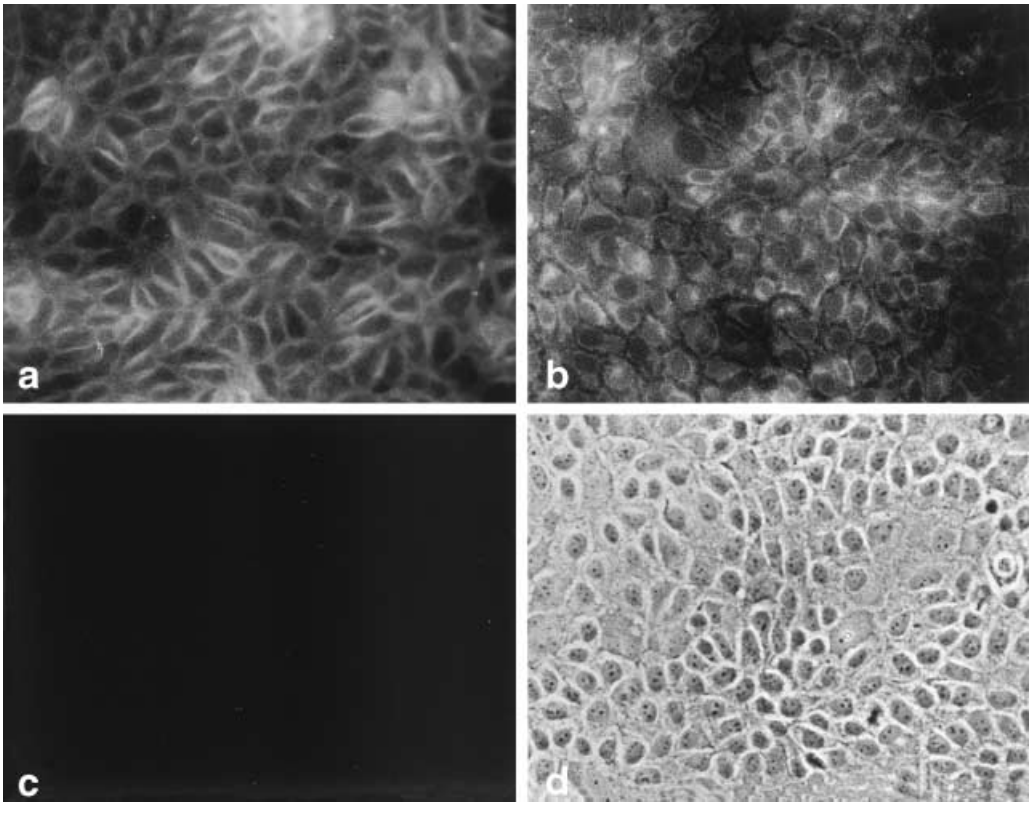

Figure 3. Immunocytochemistry of LA7 cells for growth factors. (a) Fluorescent image of cells incubated with rabbit anti-TGFα. (b) Fluorescent image of cells incubated with rabbit anti-bFGF. (c) Fluorescent image of cells incubated with rabbit pre-immune serum and the FITC-conjugated second stage antibody. (d) Phase contrast image of cells in field (c). 200 ×.

vivo are also presented to these cells in culture. The cultured cells form desmosomes, tight junctions and adherens junctions with their neighbours (Ehmann *et al*. 1998), just as *in vivo* (Monaghan & Moss 1996; Nguyen & Neville 1998; Locke *et al*. 2000; Nanba, Nakanishi & Hieda 2001; Nguyen, Parlow & Neville 2001; Runswick *et al*. 2001). Cultured MMEC express the keratins and connexins of luminal cells of the mammary ducts. In the mammary gland, as in other epithelial tissues, a mechanism must exist for maintaining an intact epithelial cell sheet by proliferation of cells to fill in for wounded or dying cells. In culture, dying LA7 cells being replaced by MMEC provides a model for maintenance of epithelial integrity. Together, these features suggest that the cells *in vitro* form an epithelial tissue similar to that found *in vivo*.

The growth factors operating in this culture system are known to be important *in vivo*. Insulin and transferrin, both components of the culture medium used here, are necessary for mammary cells *in vivo* (Li *et al*. 1998). Both TGFα and bFGF, produced by LA7 cells, are active in the mammary gland. TGFα is present in terminal end buds of developing mammary glands (Smith *et al*. 1989; Liscia *et al*. 1990, Snedeker *et al.* 1991). In adults it is produced mostly by the myoepithelial population (Smith *et al*. 1989), but is also detectable in glandular and ductal mammary cells (Yasui *et al*. 1992). It belongs to a family of growth-stimulating peptides that

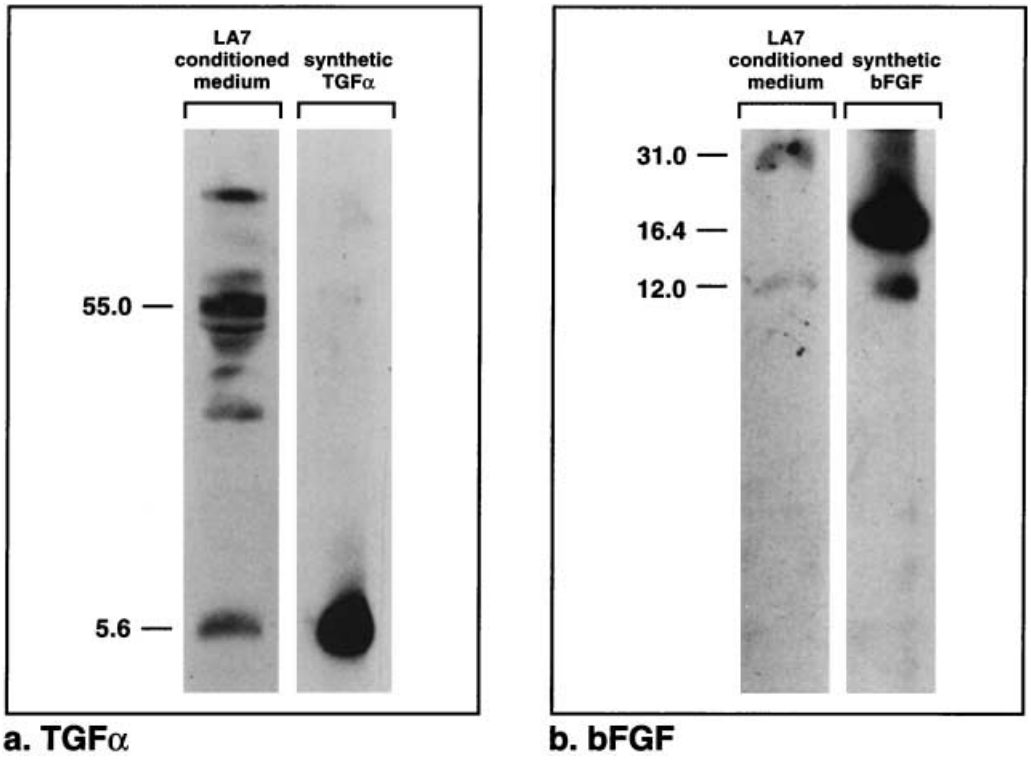

Figure 4. Growth factors from LA7 conditioned medium separated by SDS–PAGE. (a) Proteins reacting with antibodies to TGFα. One band co-migrates with the synthetic TGFα marker, and several bands are evident at higher molecular weights. (b) Proteins reacting with antibodies to bFGF. One faint band co-migrates with a minor band of the bFGF standard, and another band of ~31 kDa is visible.

includes epidermal growth factor (EGF), cripto-1, amphiregulin, heregulin α and β, and β-cellulin. Several of these are known to affect mammary cell proliferation, and more of these factors are produced in malignant than in normal tissue (Normanno *et al*. 1994; Qi *et al.* 1994; Li *et al*. 1998). The EGF receptor, to which most growth factors in this family, including TGFα, bind, is overexpressed in 50–60% of human breast tumours (Salomon *et al*. 1990). It has intrinsic tyrosine kinase activity and is located on the basolateral surfaces of many types of epithelial cells. One response to oestrogen stimulation in the mammary gland is secretion by epithelial cells of TGFα, which binds to receptors of the secreting or neighbouring cells, resulting in proliferation (Liu *et al*. 1987). TGFα precursors (~20 kDa) on one cell can directly stimulate the EGF receptor on neighbouring cells, even without cleavage of the TGFα itself (5.6 kDa) from the precursor (Brachman *et al*. 1989; Wong *et al*. 1989; Higashiyama *et al.* 1995). Thus, unsecreted TGFα can be at least as important in cell stimulation as the secreted form.

It is worth noting that others have demonstrated that radiation of a human mammary cell line resulted in increased TGFα transcription (Schmidt-Ullrich *et al*. 1992), and that TGFα-containing medium from irradiated cells activated the EGF receptor pathway on unirradiated cells (Dent *et al*. 1999). Perhaps irradiation of LA7 cells, as in this study, also stimulates their TGFα production.

bFGF, a member of the fibroblast growth factor family (Rubin *et al.* 1989; Manetti *et al.* 2000; Powers *et al.* 2000), is expressed in all cells of growing neonatal mammary ducts at all

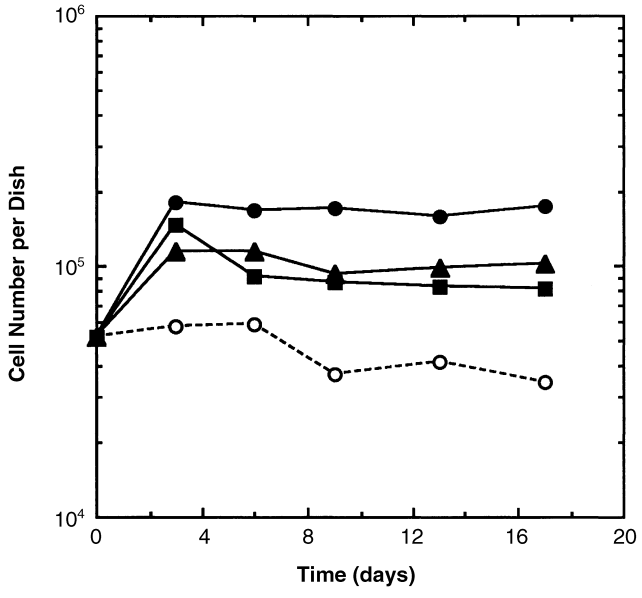

Figure 5. MMEC proliferation stimulated by growth factors. (○) Control culture medium with insulin, transferrin, and 0.5% new-born calf serum. (▲) Control medium plus 10 ng/ml TGFα (■) Control medium plus 50 ng/ml bFGF. (●) Control medium plus TGFα and bFGF. The control curve without TGFα and bFGF differs significantly from the other curves at $P < 0.05$, as does the curve with both TGFα and bFGF. The curves representing cell numbers from cultures to which either TFGα or bFGF were added do not differ significantly from each other. The results are from one experiment which is representative of three experiments.

stages of development (Rudland *et al.* 1993; Jackson *et al.* 1997; Lavendero *et al.* 1998), but in adults is secreted mostly by the myoepithelial cell population (Fernig *et al*. 1990; Gomm *et al*. 1991; Li *et al*. 1998). Myoepithelial cells also express bFGF receptors and synthesize DNA in response to bFGF (Barraclough *et al*. 1990; Gomm *et al*. 1991; Fernig *et al*. 1993; Ke *et al*. 1993; Rudland *et al*. 1993). bFGF is probably delivered to receiving cells by the extracellular matrix, where much of it is found in the mammary gland (Li *et al*. 1998; Barraclaugh *et al*. 1990). Others have demonstrated that mammary cells *in vitro* respond to bFGF and that this response is additive to that of EGF (Levay-Young *et al*. 1989), similar to our experience that synthetic bFGF and TGFα stimulate MMEC proliferation in an additive manner. In our experiments, the cells did not continue to respond to the growth factor stimuli after a few days. The same lack of continued stimulation occurred with conditioned medium from MDA-MB-231 cells (Fig. 2), which are known to secrete TGFα. Whether the growth factor receptors are downregulated or whether additional factors are required for continued stimulation is not known. Also, the culture conditions may not perfectly simulate the *in vivo* environment. For example, we were not able to detect oestrogen receptor mRNA in cultured MMEC (data not presented), even though the cells later multiplied to form glandular structures *in vivo* (Ehmann *et al*. 1987). Thus, the ability to respond to oestrogen was retained by the cultured cells even though they did not respond in the culture environment.

That LA7 cells secrete both TGFα and bFGF, as we demonstrated, suggests that they are part of the growth stimulus in LA7-conditioned medium. We also observed bands of higher molecular weights than purified TGFα and bFGF upon immune blotting of LA7 conditioned medium. It is not unusual for proteins to be secreted in forms different from those predicted. For example,

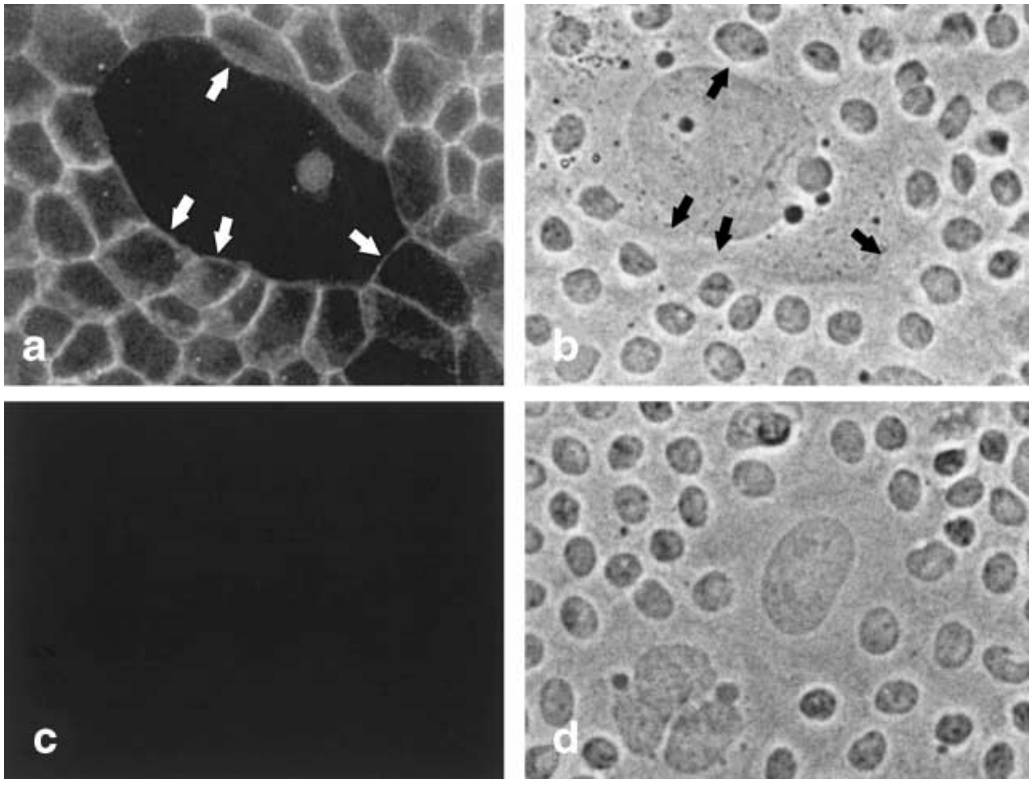

Figure 6. Adherens junctions between MMEC and an irradiated feeder LA7 cell. (a) Fluorescent image of cells incubated with antibody to E-cadherin. Adherens junctions line borders between MMEC on the edges of the field. Arrows in (a) and (b) point to sections of the junctions that line the border between MMEC and the large LA7 feeder cell in the centre of the field. (b) Phase contrast image of cells in (a). (c) Fluorescent image of a field of cells incubated with control rat serum. (d) Phase contrast image of cells in (c). 200 ×.

two rat mammary cell lines secrete bioactive TGFα of 50 kDa as well as at 5.6 kDa (Li *et al*. 1998), and a human line secretes TGFα of 7 and 30 kDa (Bates *et al*. 1988). Several human mammary lines secrete bFGF of 18, 24, 26, and 27 kDa (Li & Shipley 1991; Ke *et al*. 1993). These higher molecular weight forms might represent post-translational modification of the secreted factors or may represent antibody cross-reaction to unrelated or related protein species.

Physical contact seems to be of paramount importance in growth stimulation of MMEC (Fig. 2 and Ehmann 1992). Whether this juxtaposition of cells facilitates cell multiplication by changing the physical conformation of MMEC, whether it facilitates the action of membrane bound growth factors on LA7 cells (Brachman *et al*. 1989; Wong *et al*. 1989; Higashiyama *et al*. 1995), or whether it merely subjects the MMEC to high concentrations of growth factors secreted by LA7 cells is not clear. The importance of 'tensegrity' as a growth stimulus has been discussed (Huang & Ingber 2000), and direct transmission of growth factors, especially TGFα, from one cell membrane to another, has been well documented (Brachman *et al*. 1989; Wong *et al*. 1989; Higashiyama *et al*. 1995). Each of these modes of cell stimulation could contribute to a system that ensures replacement of a wounded cell in an epithelial sheet. That irradiated LA7 cells also support multiplication of normal mammary cells from the rat (Ehmann *et al*. 1991), the same species from which the LA7 cell line was derived, supports this model of cell replacement.

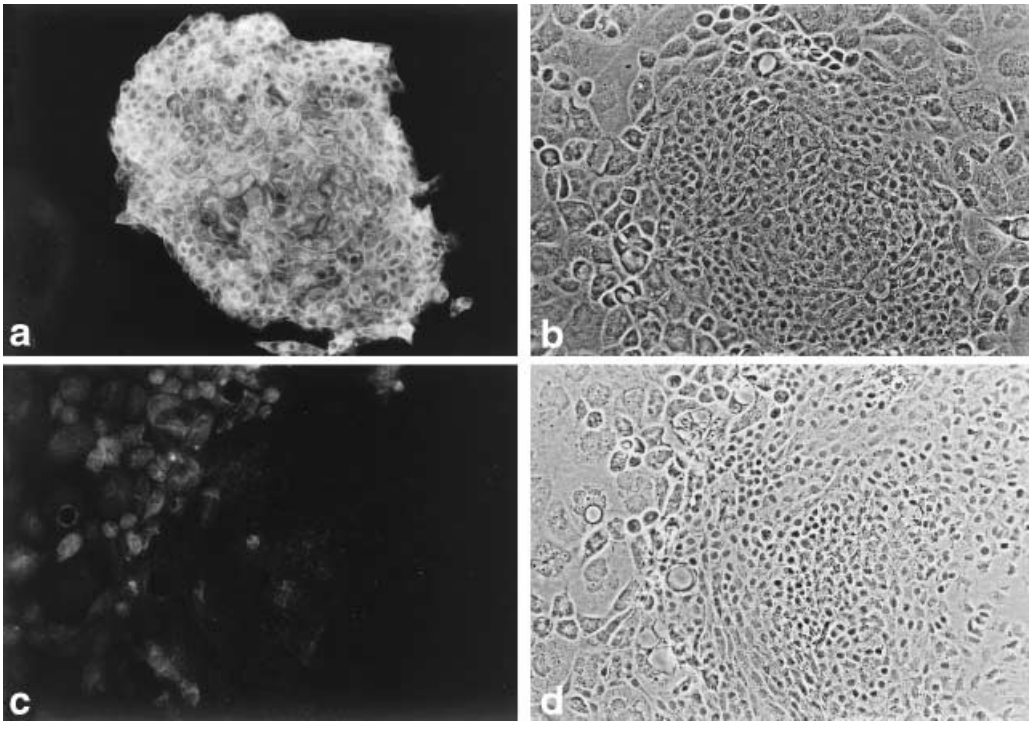

Figure 7. Keratin 8 in MMEC. (a) Fluorescent image of an MMEC colony surrounded by dying feeder LA7 cells, after exposure to a rat anti-keratin 8 serum and visualized with an FITC-conjugated antibody to rat IgG. (b) Phase contrast image of field (a). (c) Fluorescent image of control cells exposed to pre-immune rat serum. (d) Phase contrast view of field (c). An MMEC colony is on the right and feeder cells on the left. 100 ×.

Cultured MMEC express markers of the luminal ductal population, which are thought to comprise the main proliferative population of the mammary gland. *In vivo*, some of these differentiate in stages to myoepithelial cells that surround the ducts (Li *et al*. 1998). Cultured MMEC express keratins 8 and 18, and epithelial antigen, which mark luminal ductal epithelial cells *in vivo*. They do not express keratin 14 or vimentin, markers of myoepithelial cells (Stampfer *et al*. 2002). Cultured MMEC express gap junction proteins Cx26 (Ehmann *et al.* 2000), which is known to connect luminal cells *in vivo*, and Cx43, which has so far been described connecting rodent myoepithelial cells (Pozzi *et al*. 1995). Therefore, MMEC represent the *in vitro* counterpart of luminal ductal cells *in vivo* with the possibility that some of them have undergone small steps towards differentiation into myoepithelial cells. LA7 cells, on the other hand, express markers of myoepithelial cells, namely, keratin 14 and vimentin, as well as Cx43. In addition, LA7 cells produce growth factors known to be produced by myoepithelial cells *in vivo* (Gomm *et al*. 1991; Li *et al*. 1998).

In summary, MMEC and LA7 cells together in culture seem to form an epithelial tissue comparable to that found *in vivo*. The MMEC respond to growth factors produced by LA7 cells, as luminal epithelial cells *in vivo* respond to growth factors made by myoepithelial cells (Li *et al*. 1998). The cells are all in contact with each other and connected by junctions, as in the mammary gland. The MMEC grown in this culture system carry markers of normal luminal cells of mammary ducts. Because this culture system stimulates extensive proliferation and maintains cells in their normal state, it provides an important tool for studying normal cell–cell interactions that have significance *in vivo*.

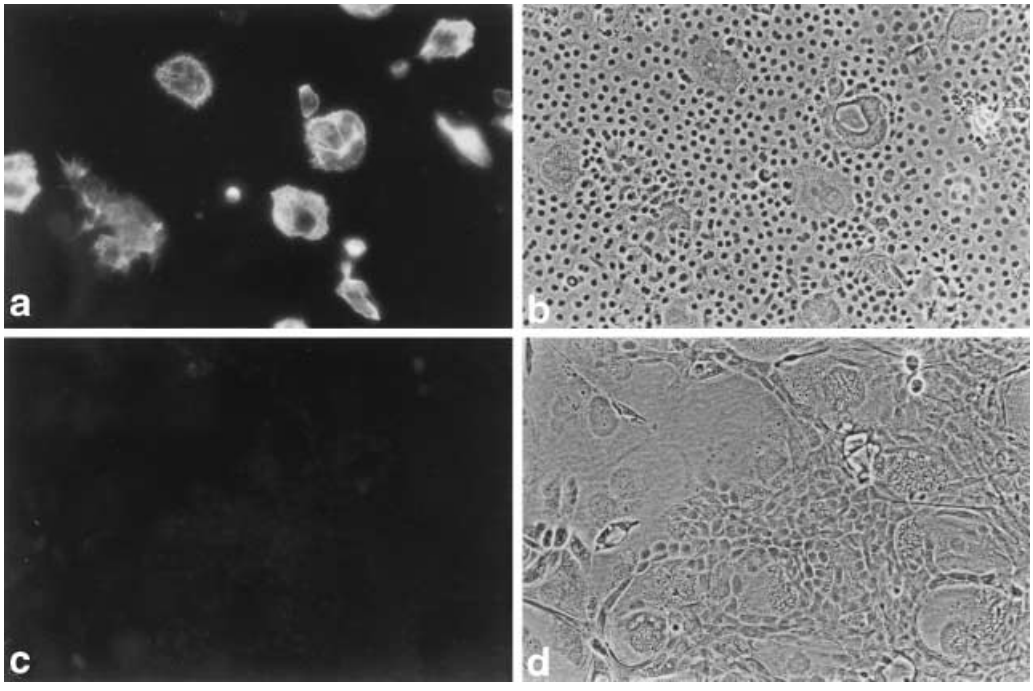

Figure 8. Vimentin in LA7 feeders. (a) A field of nearly confluent MMEC which surround a few remaining LA7 feeders, exposed to mouse anti-vimentin serum. The LA7 cells, but not the MMEC, stain brightly. (b) Phase contrast view of field (a). (c) Control field of cells exposed to mouse pre-immune serum. (d) Phase contrast view of field (c). 100 ×.

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