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# Maintenance of normal human breast organoids within rat mammary fat pads in organ culture

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**Summary.** Normal human breast organoids, derived by collagenase digestion of reduction mammaplasty tissue specimens, have been cultured in vitro for up to 28 days after injection into organ cultures of virgin rat mammary fat pads. The culture medium was serum-free Waymouth's MB 752/1 with hormonal additives. The rat mammary tissue responded well to growth-promoting and lactogenic stimuli in the culture medium, in agreement with previous investigations. Using immunohistochemistry casein was identified in rat epithelia exposed to lactogenic medium. Human organoids in culture remained viable but did not show hormone-responsiveness. Electron microscopy confirmed the presence of both luminal epithelial cells and myoepithelial cells.

The serum-free culture of normal human breast organoids in a three-dimensional matrix provides a system in which to study factors controlling growth and differentiation.

**Key words:** Human – Rat – Breast – Mammary

## Introduction

Many approaches have been made to study normal human breast tissue in in vitro culture systems. Normal human breast epithelium has been maintained in organ culture for several months in a medium containing serum (Hillman et al. 1980), but only for a few days in serum-free medium (Flaxman 1974; Strum and Hillman 1981). Thus studies of the hormone dependence of mammary growth and differentiation have been confined to these short term cultures. Normal human breast epithelium prepared by collagenase digestion can be used to establish monolayer cultures (Hallowes et al. 1980). In these cultures luminal membrane antigens are expressed (Easty et al. 1980; Edwards et al. 1986) but organisation of the epithelium is not observed. It is likely that monolayer cultures of breast fail due to the absence of a three-dimensional supporting matrix. Levine has attempted to supply an appropriate support for rat mammary epithelial cells in culture using an adipocyte cell line as a feeder layer (Levine and Stockdale 1984), but this type of system still does not provide a three-dimensional structure. In vitro studies using human breast epithelium embedded into a matrix of rat-tail collagen have demonstrated the retention of some organisation (Foster et al. 1983). The only system where human breast tissue has been demonstrated to undergo a lactational response is when implanted into the nude mouse (Gusterson et al. 1984). This observation suggests that the stromal environment is of importance for hormonal responsiveness.

The ability of the rat mammary fat pad in vitro to provide a matrix for human breast tissue has therefore been examined as it also provides a system where the hormones can be manipulated in a controlled manner.

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## Methods

*Preparation of tissue.* Three to four week old female rats were killed by cervical dislocation, and the second and third thoracic and inguinal mammary glands were immediately removed using aseptic dissection techniques (Rivera 1971). The glands were cut into pieces about 40 $mm^3$ and attached to dry, sterile Nucleopore membranes (13 mm diameter, 8 μ pore size – Sterilin, Feltham, U.K.) and placed in 199 tissue culture medium (Gibco, Uxbridge, U.K.) containing fungizone (2.5 μg/ml), penicillin (50 U/ml), streptomycin (50 U/ml) and 10 mM Hepes (adjusted to pH 7.3 with 2 M sodium hydroxide) in a petri dish.

Normal human breast organoids were prepared from a total of 16 reduction mammaplasty specimens using a previously described method (Foster et al. 1983).

**Table 1.** Concentrations of various hormones in the different media used for culture of mammary fat pads

| Hormone additions | Type of medium | | |
|---|---|---|---|
| | Maintenance | Growth-promoting | Lactogenic |
| Insulin | 5 μg/ml | 5 μg/ml | 5 μg/ml |
| Aldosterone | 1 μg/ml | 1 μg/ml | – |
| 17β-oestradiol | – | 1 μg/ml | – |
| Progesterone | – | 1 μg/ml | – |
| Hydrocortisone | – | – | 5 μg/ml |
| Prolactin (ovine) | – | 5 μg/ml | 5 μg/ml |
| Growth hormone (ovine) | – | 5 μg/ml | 5 μg/ml |

*Organ culture.* Waymouth's MB 752/1 medium (Gibco Europe, Glasgow, Scotland was prepared by addition of fungizone (2.5 μg/ml), penicillin (50 U/ml), streptomycin (50 U/ml) and various combinations of hormones to the final concentrations indicated in Table 1. These hormone combinations were derived from Banerjee et al. 1976; Prop 1981; Ichinose and Nandi 1966; Wood et al. 1975. Stock solutions of steroid hormones were prepared by taking up the weighed steroid in a small volume of ethanol and diluting with sterile 0.18 M saline to working concentrations of 1 mg/ml (progesterone and aldosterone – both from Sigma St. Louis, MO, USA) and 1 μg/ml (17β-oestradiol – Sigma). Prolactin (ovine from Sigma) and growth hormone (ovine from NIH) were taken up in a small volume of sterile 0.001 N sodium hydroxide and diluted with Waymouths medium to a working concentration of 1 mg/ml. Insulin (bovine from Sigma) was taken up in sterile 0.05 N hydrochloric acid and diluted with medium to a working concentration of 1 mg/ml. Protein hormone solutions were filtered (0.45 μ Millipore).

Sterile supporting stainless steel grids (mesh approx. 0.6 mm) were placed in 12 well Linbro tissue culture plates (Flow Laboratories, Irvine, Ayrshire, U.K.) and 2 ml of complete Waymouths medium was pipetted over the grids. Each rat mammary fat pad was injected with 0.02–0.05 ml breast organoids (approximately 10–30) in Hepes-buffered DMEM – Gibco, and placed on the grid with the Nucleopore membrane in contact with the grid and the medium. The cultures were placed in a humidified atmosphere of 95% oxygen, 5% carbon dioxide at 37° C. Every 3 or 4 days each grid was transferred (with tissue) to a new 12 well tissue culture plate containing 2 ml of fresh medium, and the incubation was continued. At the end of the 14 or 28 day culture period the tissue was fixed overnight in methacarn (6:3:1; methanol:1,1,1-trichloroethane:glacial acetic acid). Some tissue was fixed for electron microscopy using 2% glutaraldehyde in 0.05 M sodium phosphate buffer, pH 7.3, at 4° C for 24 h. Tissue was post-fixed and embedded as previously described (Foster et al. 1983).

*Whole mount preparation of cultured tissue.* Pieces of tissue fixed in methacarn were rinsed in running water and stained in Deldefield's haematoxylin (Raymond Lamb, 6 Sunbeam Road, London, N.W.10) for 1 h and then rinsed in water for 30 min. Destaining was carried out overnight in acid alcohol (2% 1M HCl in 70% ethanol) followed by rinsing in running water for 1 h. The tissue was then dehydrated through graded alcohols (3 h in 95% ethanol, 6 h in 99% ethanol and 8 h in 100% ethanol) clarified and stored in methyl salicylate (Sigma). Whole mounts were examined under the dissecting microscope.

*Immunocytochemistry.* Fixed tissue was processed through graded alcohols and xylene, and then embedded in paraffin wax. Tissue which had been whole mounted was washed free of methyl salicylate by soaking for 24 h in 100% ethanol, and then embedded in paraffin wax.

Serial, 5 μm, sections were cut onto glass slides, dewaxed with xylene, and taken to water through ethanol. Sections were stained with haematoxylin and eosin or by immunohistochemistry using the antibody LICR-LON-M8 (method and specificity described previously (Foster et al. 1982) and an anti-casein monoclonal antibody 32.2 (Earl and McIlhinney 1985). Sections were counterstained in Meyer's haemalum, and mounted in Hydromount.

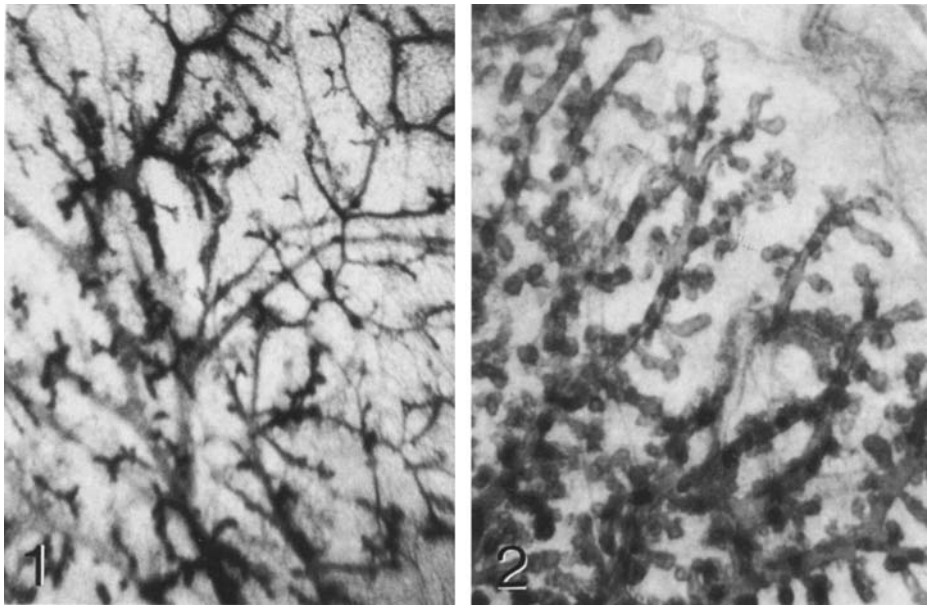
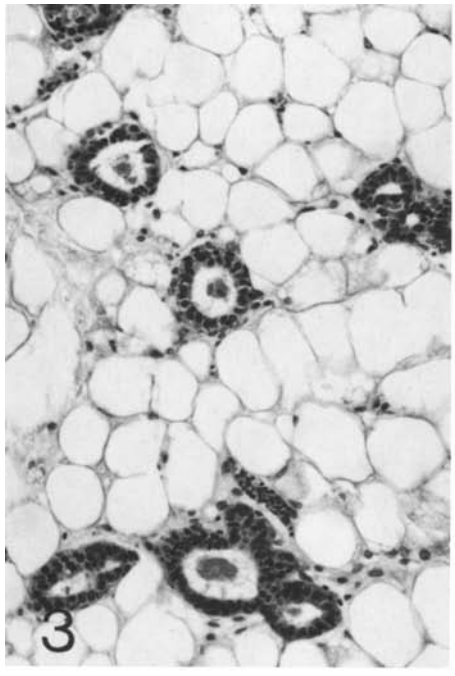
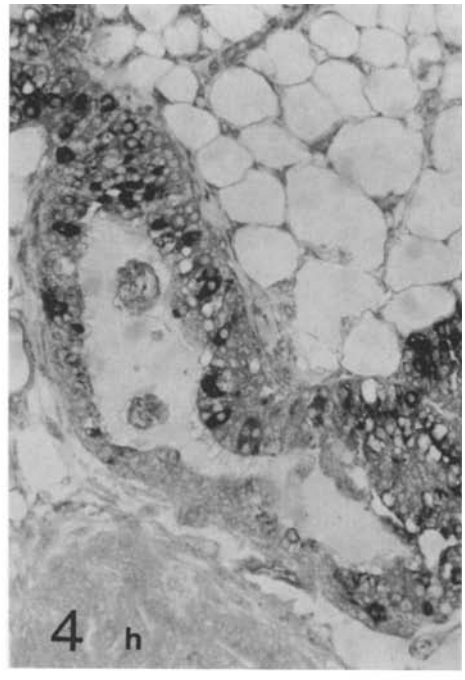
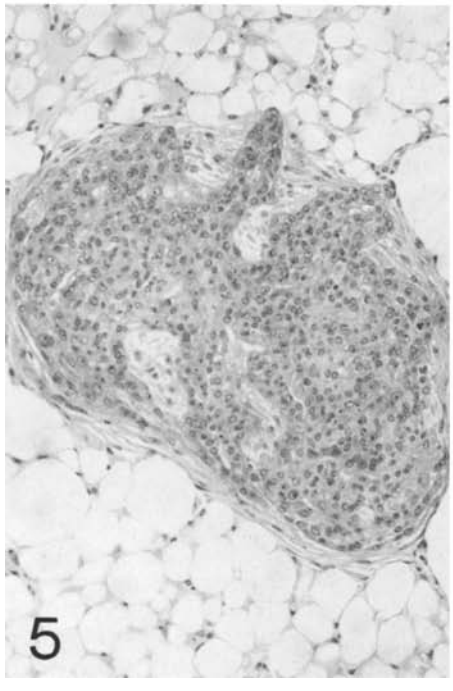



**Fig. 1.** Whole mount of rat mammary fat pad dissected from 3–4 week old virgin and immediately fixed in methacarn. Magnification ×3.6

**Fig. 2.** Whole mount of 3–4 week old virgin rat mammary fat pad incubated in lactogenic culture medium for 14 days resulting in the growth and distension of the mammary ducts. Magnification ×3.6



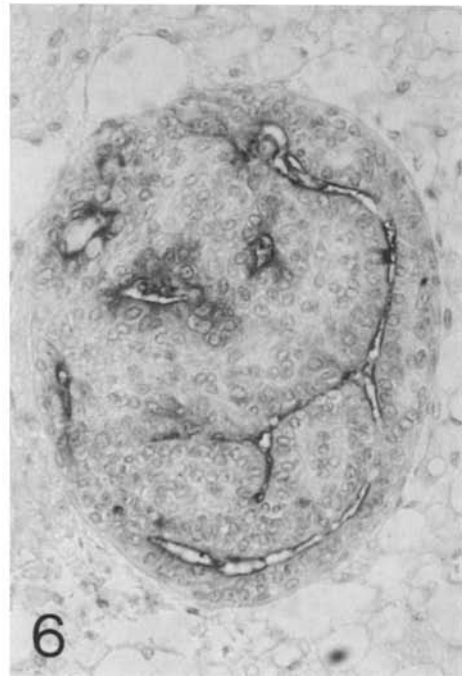




**Fig. 3.** 3–4 week old virgin rat mammary fat pad stained with haematoxylin and eosin. Magnification ×157

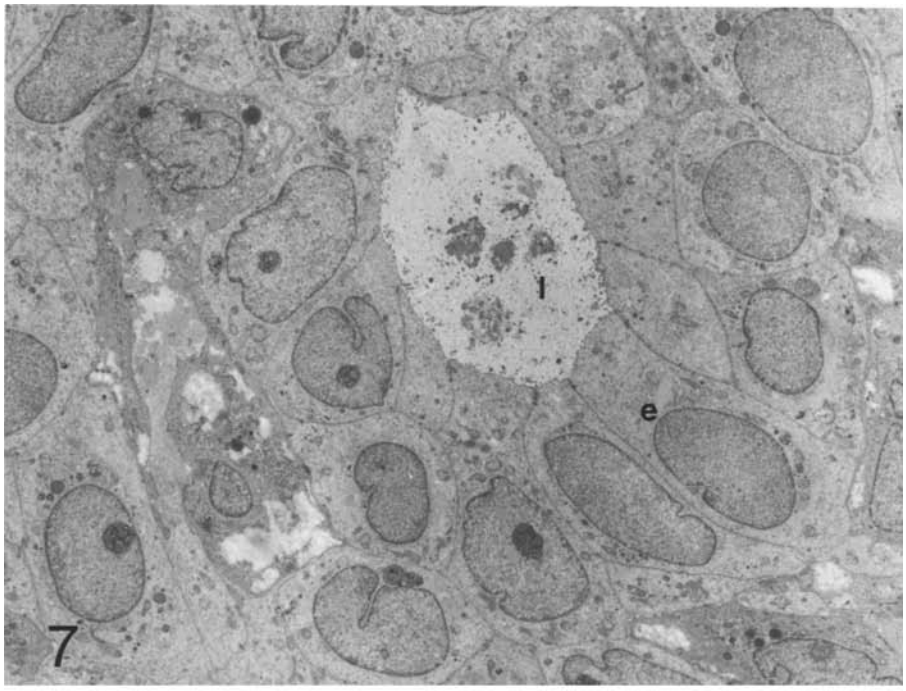
**Fig. 4.** 3–4 week old virgin rat mammary fat pad cultured in lactogenic medium showing secretory activity, and staining with casein antibody in the rodent tissue. Human tissue (*h*) is unstained. Magnification ×175

**Fig. 5.** Human breast organoids growing within rat mammary fat pads cultured in lactogenic medium. Note outgrowths of human cells into the rat stromal tissue. Magnification ×175

**Fig. 6.** Human breast organoids growing within rat mammary fat pads cultured in lactogenic medium and stained with monoclonal antibody LICR-LON-M8; note apical membrane staining. Magnification ×300

**Table 2.** Success and viability of rat mammary fat pads containing injected human breast organoids

| Type of medium | Total no. of fat pads | No. containing human tissue | % in which rat mammary epithelium viable | % in which human mammary epithelium viable | % in which human tissue shows organization |
|---|---|---|---|---|---|
| Maintenance | 31 | 19 | 79 | 74 | 68 |
| Growth-promoting | 18 | 15 | 43 | 67 | 33 |
| Lactogenic | 31 | 31 | 64 | 90 | 68 |



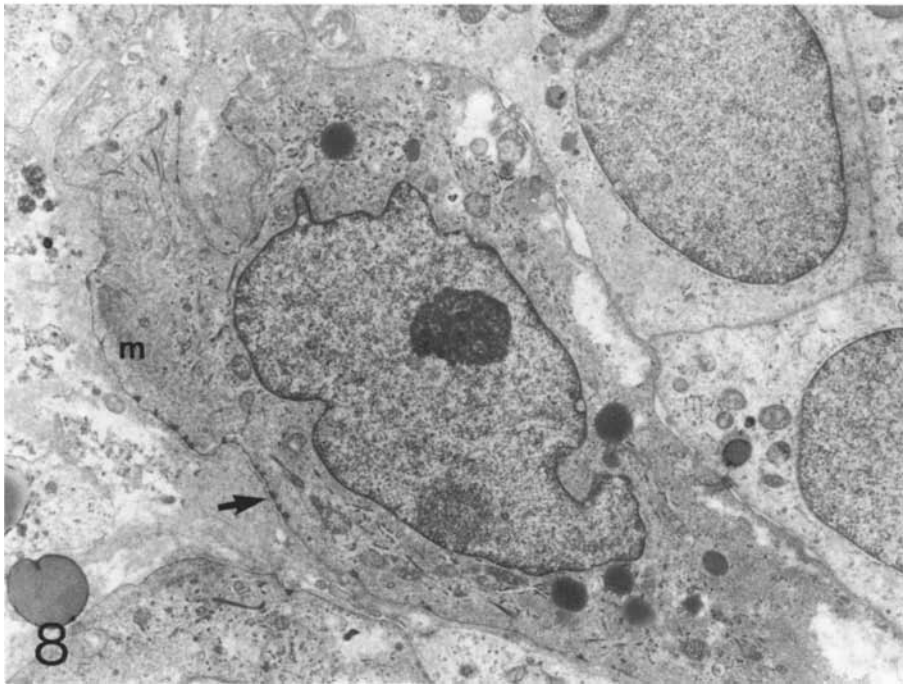




**Fig. 7.** Electron micrograph of human breast organoids cultured within rat mammary fat pads in lactogenic medium. Note the lumen (*l*) bordered by epithelial cells (*e*). Magnification ×2,730

**Fig. 8.** Electron micrograph of myoepithelial cell of human breast organoids cultured within rat mammary fat pads in lactogenic medium. Characteristic features of myoepithelial cells can seen; Hemidesmosomes (*arrow*), myofilaments (*m*). Magnification ×7,435

## Results

### *Rat mammary tissue*

At the start of the culture, the morphology of the mammary tree was essentially ductal with some end buds (Fig. 1). Large areas of stroma could be seen between the mammary parenchyma. Culture in maintenance medium (insulin + aldosterone) resulted in viable mammary epithelium, but there was no significant growth. After incubation in lactogenic or growth promoting medium (Table 1) for between 14 and 28 days an extensive lobulo-alveolar development was induced (Fig. 2). The extent of epithelial growth into the adipose tissue can clearly be seen by comparison of Figs. 1 and 2. In the two different media growth was similar but the mammary ducts and end buds appeared more

distended in lactogenic medium. This was confirmed histologically where the resting ducts (Fig. 3) had expanded to produce cellular alveoli some of which stained for casein after incubation in the lactogenic medium (Fig. 4). Secretory droplets and luminal material could be clearly seen in histological sections of tissue cultured in lactogenic medium. There was no significant difference in the degree of differentiation observed at 14 days as compared with 28 days in culture, and subsequent cultures were analysed after 14 days incubation.

*Normal human breast tissue*

The presence of human mammary tissue was identified in 80% of injected rat mammary fat pads (Table 2). The recovery of human breast tissue was lowest when incubated in maintenance medium and the viability of the injected human organoids tended to be better than the surrounding rat tissue notably in the lactational medium. In the whole mounts human tissue could also be identified which in some instances had apparent outgrowths. This was confirmed histologically where tongues of epithelial cells were observed growing out into the rat breast parenchyma with a condensation of fibroblasts at the periphery of the human elements (Fig. 5). Some epithelial organization was seen at the light microscope level with lumina positively stained using the M8 antibody (Fig. 6), which is specific for the apical membrane of human epithelial cells (Foster et al. 1982). When tissue containing rat and human epithelia was incubated in lactogenic medium, casein was only identified in the lumina and epithelia of the rat tissue (Fig. 4).

Electron micrographs of the injected human breast organoids showed that the cells were viable and well organised (Fig. 7). Fully differentiated luminal epithelial cells, characterised by their apical microvilli, junctional complexes, electron-lucent cytoplasm and many organelles polarised toward the lumen, could be identified. Basally situated myoepithelial cells were also present and were characterized by the presence of myofilaments with focal densities, micropinocytotic vesicles and hemidesmosomes adjoining the basal lamina (Fig. 8).

## Discussion

Mammary gland explants from young virgin rats have been successfully maintained in organ culture in serum-free medium and shown to be hormone responsive. The results obtained essentially agree with previous studies of mouse (Ichinose and Nandi 1966; Wood et al. 1975; Banerjee et al. 1976; Tonelli and Sorof 1980; Prop 1981) and rat (Dilley and Nandi 1968) mammary tissue where a lactogenic response was observed in incubations containing insulin, prolactin, growth hormone and cortisol. Casein was identified in this tissue in agreement with previous experiments (Emerman et al. 1977; Terry et al. 1977). Normal human breast organoids grown within rat mammary fat pads in organ culture can be maintained successfully for up to 28 days. The organoids integrate into the rodent stroma and retain the characteristic structural and functional features of mammary differentiation after 14 days in culture. The luminal epithelial membrane antigen of human breast recognised by the M8 antibody (Foster et al. 1982) was clearly present on the apical membranes of epithelial cells derived from the injected organoids. Fully differentiated epithelial cells and myoepithelial cells were observed ultrastructurally.

This system for the three-dimensional culture of organoids derived from normal human breast in a serum-free medium provides a model for the study of endocrine factors controlling lactogenesis and the importance of cell-cell interactions. The role of the three-dimensional matrix which surrounds the growing human organoid is of particular interest. A collagen matrix of non-mammary origin is also able to support growth and differentiation of the organoids in the presence of serum (Smith et al. 1985). In neither system has full lactational differentiation comparable with that in the rat or mouse been obtained, although it is known that organoids derived from human breast have the capacity to differentiate and synthesise $\alpha$-lactalbumin within 22 days when injected into the mammary pad of mice which are subsequently mated (Gusterson et al. 1984). A sequential culture in growth promoting medium followed by lactogenic medium did not mimic the effect of pregnancy in pregnant mice (H. Stewart – unpublished observation). It is likely that several other humoral agents such as epidermal growth factor (Tonelli and Sorof 1981) absent from these serum-free cultures may be required for normal growth and differentiation.

The injection of normal human breast cells into rodent mammary fat pads in organ culture provides a system which can be readily manipulated to study factors involved in mammary growth, differentiation and transformation.

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