

Regeneration of mammary glands *in vivo* from isolated mammary ducts

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SUMMARY

Rat mammary ducts, free of buds, can alone regenerate complete mammary trees when transplanted into the interscapular fat pads of syngeneic host rats. All the main mammary cell types are identified within such outgrowths. Epithelial cells, which show the presence of milk fat globule membrane antigens and microvilli on their luminal surfaces, line the ducts. Basal cells surrounding the ducts show characteristic features of myoepithelial cells: immunoreactive actin and keratin within the cytoplasm, myofilaments, pinocytotic vesicles and hemidesmosomal attachments to the basement membrane. Cells within the end buds and lateral buds, however, show few if any cytoplasmic myofilaments and are relatively undifferentiated in appearance. Intermediate morphologies between these cells and myoepithelial cells are seen nearer the ducts. In this respect they exactly resemble the cap cells found in terminal end buds (TEBs) of normal mammary glands. Occasional epithelial cells within alveolar buds show the presence of immunoreactive casein, which is a product of secretory alveolar cells in the normal rat mammary gland. Dissected terminal end buds can regenerate similar ductal outgrowths. Thus, ductal tissue alone can generate all the major mammary cell types seen in the normal gland, including the cap cells.

INTRODUCTION

The rat mammary gland arises embryologically as a rounded bud of epithelial cells. This mass develops to form a branching system of ducts which is present even within neonatal animals (Anderson, 1978). In the developing gland of the virgin rat the ducts terminate in terminal end buds, which are responsible for ductal elongation. The spaces between branching ducts are occupied by smaller buds that branch off laterally from the main ductal system. Both types of bud divide to form small bud-like clusters called alveolar buds. The alveolar buds enlarge and develop into mature alveoli during pregnancy and become functionally active, secreting milk products including casein, during lactation (Turner & Gomez, 1933; Russo & Russo, 1978). The buds that are found in the developing rat mammary gland are composed of undifferentiated cap cells and large clear cells in addition to the

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epithelial and myoepithelial cells that line the ducts (Williams & Daniel, 1983; Ormerod & Rudland, 1984).

In order to study the morphogenetic capacity of cells within the mammary system, parts of the mammary gland have been dissected out and transplanted to other suitable sites in syngeneic animals. The transplanted material may be monodispersed cells (Daniel & DeOme, 1965; Gould, Biel & Clifton, 1977; Sinha, 1981), partially digested glands or organoids (Faulkin & DeOme, 1960), or whole segments of glands (Hoshino, 1962, 1963; Williams & Hoshino, 1970). Suitable transplantation sites include mammary fat pads cleared of parenchymal tissue (Sekhri *et al.* 1967), the interscapular fat pad (Gould *et al.* 1977), the subcutaneous dorsal area (Hoshino, 1963), the pararenal fat pad (Hoshino, 1963) and underneath the kidney capsule (Sakakura *et al.* 1979). In all cases fully developed mammary glands are generated which will lactate within isologous, pregnant hosts (Hoshino, 1963, 1964). We have examined the potential of cells within (1) bud-free ducts and (2) terminal end buds (TEBs) to regenerate the cell types and structures seen in the normal rat mammary gland. The mammary cells within the resultant outgrowths have been identified by immunocytochemical and electron microscopic techniques. In particular these techniques enable us to investigate the cell types within the developing buds of the regenerated outgrowths. We can compare such structures to those found and recently described *in vivo* (Williams & Daniel, 1983; Ormerod & Rudland, 1984). We have utilized vital staining of the rat mammary gland (Ormerod, 1983) to identify specific regions of the parenchymal tissue. These specific regions have then been dissected out and grafted into the interscapular fat pad of syngeneic hosts (Gould *et al.* 1977). This site was chosen because of its convenience and because there is no likelihood of regrowth of the host's mammary tissue. Using these techniques we aim to find out whether the regenerative and differentiating capacity of the mammary gland is confined to developing buds or whether normal ductal tissue can produce mammary outgrowths when stimulated to grow in a suitable environment.

MATERIALS AND METHODS

Animals

Animals used were female Ludwig Wistar OLA rats of varying ages (see Table 1) from OLAC, Banbury, Oxfordshire. Rats were housed in M2 cages, six animals per cage, and were maintained in a temperature of 70–72°C in natural light and darkness. They were fed on expanded Rat and Mouse Diet No. 1 (B.P. Ltd, Witham, Essex) and water *ad libitum*. Rats were injected subcutaneously in the inguinal fat pad with a 0.25% (w/v) methylene blue solution. The following day, the animals were killed by cervical dislocation. A ventral incision was made, and the two flaps of skin peeled back from the body wall so that the inguinal mammary glands were exposed. Methylene blue stained the mammary parenchyma, but the fat pad remained white. Under a dissecting microscope the fat surrounding the mammary parenchyma was partially removed and pieces of mammary parenchyma cut out using fine forceps and spring scissors. Ducts that were free of lateral buds and TEBs were dissected out. Recipient rats were anaesthetized using ether. The area of skin at the back of the neck was

shaved and a small incision made between the two shoulder blades. A portion of the interscapular fat pad was pulled through the incision and injected with dissected pieces of mammary gland using a trochar. The fat pad was allowed to fall back into place and the incision was sealed using staples.

Histology and immunocytochemistry

At the end of the experiment rats were killed using an overdose of ether and the skin at the back of the neck was shaved, and cut so as to expose the interscapular fat pad. The fat pad was peeled away from the skin and stretched out on a glass microscope slide. Fat pads were then placed in methacarn fixative (10 % glacial acetic acid, 30 % inhisol, 60 % methanol) overnight (Warburton, Mitchell, Ormerod & Rudland, 1982). The following day fat pads were stained by immersion in 0.5 % methylene blue solution for 1 h. After rinsing in ethanol the fat pads were examined under a microscope, and areas of ductal growth were photographed and then dissected out for histological examination. Such samples were embedded in paraffin wax and immunocytochemically stained by the indirect alkaline phosphatase procedure as described previously using rabbit antisera to human keratins, pig actin, rat milk fat globule membranes (MFGM), rat casein, laminin and fibronectin (Warburton *et al.* 1982; Rudland, Hughes, Twiston Davies & Warburton, 1983; Ormerod & Rudland, 1984). Sections were counterstained with the nuclear stain, haemalum. Photographs were recorded on Ilford Pan F film using a Reichert Polyvar microscope equipped with a green filter. Each type of structure present within the implants was examined in at least 20 sections from different fat pads. Immunocytochemical staining was carried out on at least four sections from each group of rats (Table 1) and the percentage of cells staining was measured.

Electron microscopy

Interscapular fat pads were dissected out, stretched on glass microscope slides and fixed in 2 % (w/v) glutaraldehyde for a minimum of 24 h, at 4°C. The fixative was phosphate buffered (0.05 M, pH 7.4) and the osmotic pressure was adjusted to 350 mosm by addition of sucrose. Fat pads were then transferred to 0.1 M-phosphate buffer (pH 7.4) for 1–3 days (Strum, 1979). After further fixation in acetone for 24 h the whole mounts were stained with 0.5 % methylene blue in normal saline for 10–12 h. Whole mounts were then transferred *via* a graded series of ethanols (50 %, 70 %, 95 %: each overnight) to 100 % ethanol. During this stage the fat pads were examined under a dissecting binocular microscope and any organized structures were dissected out. The pieces were then embedded *via* propylene oxide (20 min) and 50:50 propylene oxide:resin mixture overnight. Samples were embedded in a degassed Epon Araldite resin mixture and polymerized at 60°C for 48 h.

Thick sections (1–2 µm) were cut using glass knives on a Reichert OM U4 microtome and stained with 1 % toluidine blue in 1 % borax. Selected areas were trimmed and ultrathin sections (50–70 nm) were cut using a Diatome diamond knife. Sections were stained with methanolic uranyl acetate and aqueous lead citrate and examined in a Philips EM400 at 80 kV.

RESULTS

Gross morphology and histology of implants

Six weeks after implantation of the interscapular fat pad with either bud-free ducts or TEBs, small ductal outgrowths were found in 50–60 % of the host rats (Table 1, Fig. 1A). The outgrowths showed both monopodial and dichotomous branching, with the formation of lateral branches and forked buds at the termini of the branches, respectively. Branches at the extremities of the outgrowths often had swollen, bulbous ends which resembled the TEBs found in the normal rat

mammary gland (Russo *et al.* 1977). Some of the ducts had an indented rather than a smooth outline with many lateral buds positioned along their length (Fig. 1B). All the ductal outgrowths grew apart and no ducts crossed each other (Fig. 1B).

Histological examination of outgrowths from implanted TEBs or ducts were similar and showed that many cells were organized into ductal structures containing lumina (Fig. 2). The sites of origin of the outgrowths appeared as an indistinctly outlined clump of cells in whole-mount preparations. Histological sections of these regions showed that some cells were organized around lumina. However, none were arranged in well-spaced-out ducts as in the peripheral areas of outgrowths. The ductal outgrowths comprised two or three layers of cells lining a central lumen. Lateral buds and end buds were composed of a solid mass of cells (Fig. 3A,B). End buds often contained a few small lumina within the solid cellular mass (Fig. 3A,B). Mitotic figures were frequently seen within the end buds and lateral buds in both the outer peripheral and inner or cortical cells (Fig. 2). A connective tissue sheath was virtually absent from the tips of the end buds.

In whole-mount preparations of outgrowths which had been growing in the interscapular fat for 12–16 weeks, some primary and secondary ducts terminated in indented, swollen end buds but the majority showed no such swellings and resembled more closely the terminal ducts of the mammary gland. Occasional ducts consisted of clusters of three to six smaller buds at their termini (Table 1, Fig. 1B). These latter buds were lined by two or three cell layers, and resembled morphologically the alveolar buds of the normal rat mammary gland. Occasional areas of abnormal growth of hyperplasia were identified in about 20% of the fat pads (not shown).

Immunocytochemical staining of implants

Immunocytochemical staining patterns of ducts, terminal end buds and clusters of smaller buds were identical for transplants derived from bud-free ducts and

Table 1. *Summary of outgrowths obtained with different transplants*

Number of rats	Age at transplantation (weeks)	Transplant type*	Time in host (weeks)	Outgrowth number†	Morphology‡
6	7	TEB	6	3	Ductal, LB, TEB
6	7	TEB	12	5	Ductal, LB, TEB
6	8	TEB	16	6	Ductal, LB, TEB, AB
5	7	DUCT	6	4	Ductal, LB, TEB
6	8	DUCT	10	4	Ductal, LB, TEB
6	8	DUCT	12	3	Ductal, LB, TEB
4	8	DUCT	15	3	Ductal, LB, TEB, AB

* 'TEB' indicates 4–6 terminal end buds which were removed from the subtending duct at the neck of the TEB. 'DUCT' indicates 4–6 segments of duct without any lateral buds, approximate length 200 μm .

† Number of rats with interscapular fat pads containing outgrowth.

‡ 'Ductal' indicates a branching system of ducts; LB, lateral buds; TEB, terminal end buds; AB, alveolar buds.

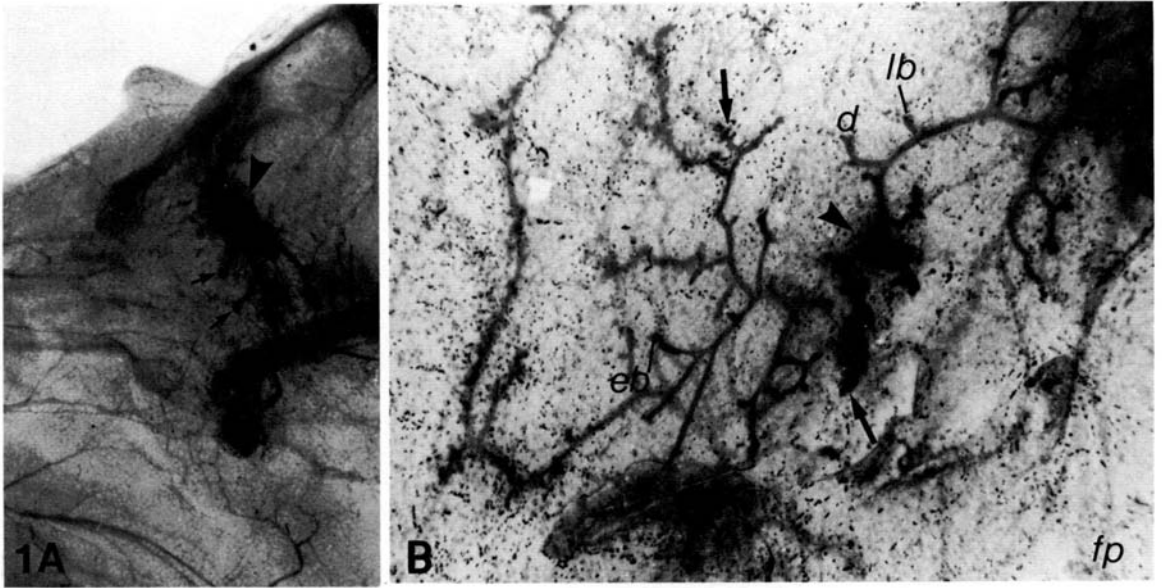


Fig. 1. Whole mounts of regenerating ductal mammary glands.

(A) 6 weeks after implantation of TEBs. Site of the outgrowth (arrowhead) appears as a large clump of cells from which three ductal branches originate. The ducts end in dilated end buds (arrows). $\times 18.8$.

(B) 16 weeks after implantation of portions of ducts within the interscapular fat pad (*fp*). Two main branching systems originate from the transplant site (arrowhead). The ducts show the presence of lateral branches (*lb*), swollen end buds (*eb*), duct (*d*) and small clusters of buds (arrows). $\times 18.8$.

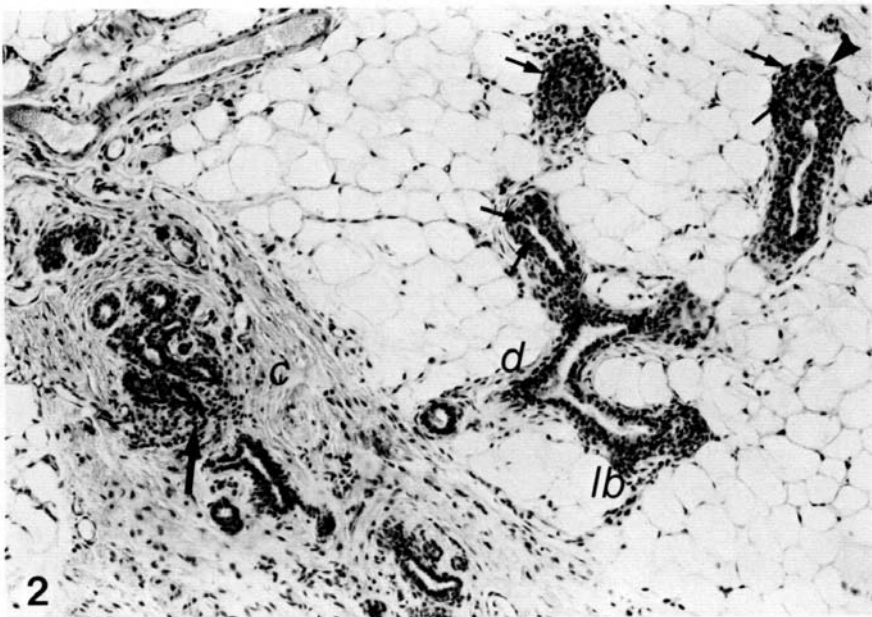
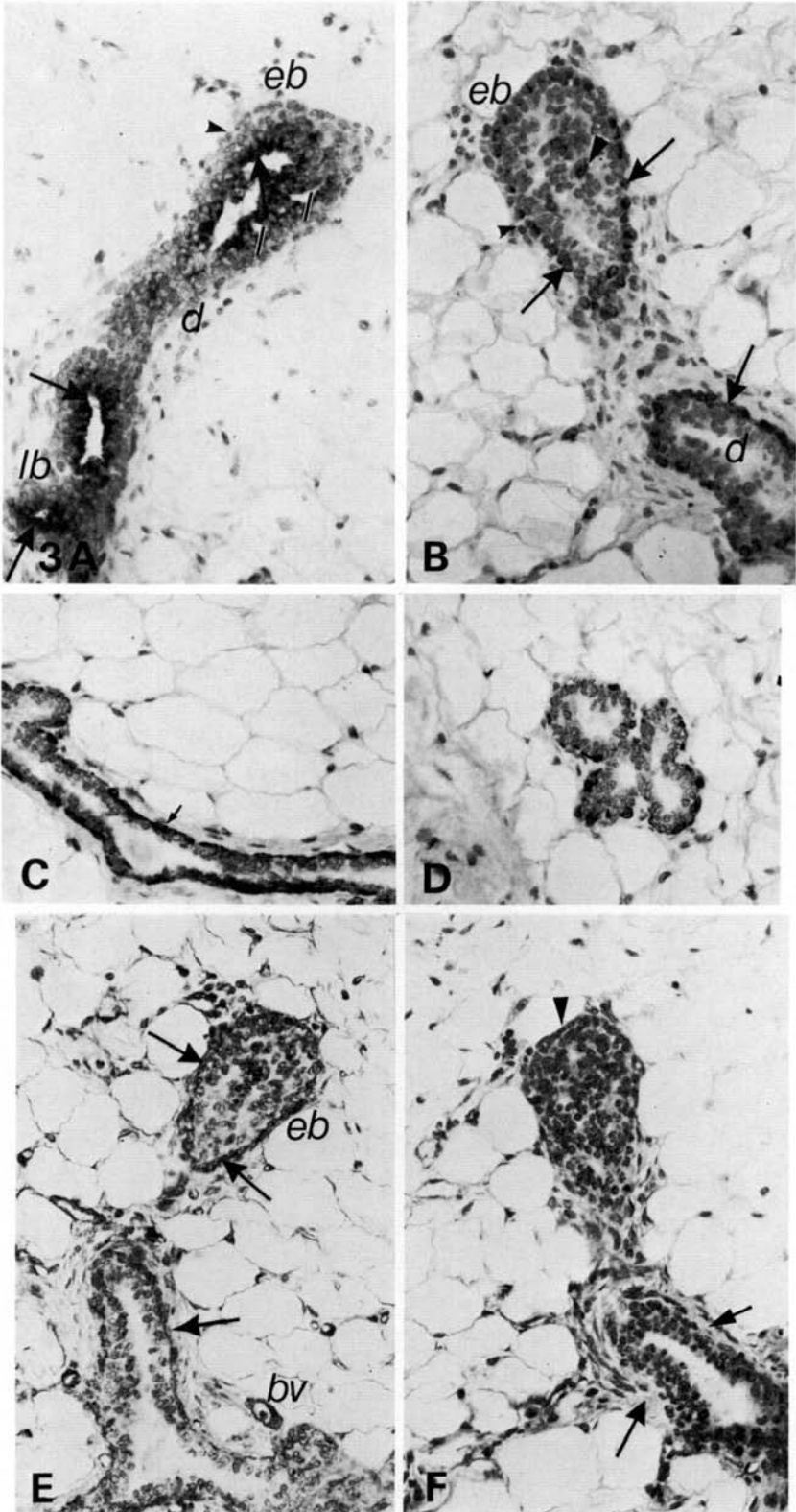


Fig. 2. Histological section of a gland regenerated from segments of ducts, after 6 weeks' growth and stained with H and E. Large arrow shows the origin of the branches, note the thick connective tissue sheath (*c*) around this area, an end bud (arrowhead), and branching portions of a duct (*d*) have grown into the host interscapular fat pad. Lateral bud (*lb*), mitotic figures (small arrows). $\times 117$.



from TEBs after 6 and 16 weeks' growth. The staining pattern of normal mammary glands with the antisera used in the present study has been described in detail previously (Warburton *et al.* 1982; Rudland *et al.* 1983; Ormerod & Rudland, 1984).

Milk fat globule membrane antigen (MFGM) was present on the luminal surfaces and within the apical cytoplasm of all the cells lining lumina (Fig. 3). In contrast, casein was located only in 10–15 % of cells lining the small clusters of buds (not shown). Within the terminal end buds and lateral buds occasional cells (2–5 %) showed staining with this antiserum. Immunoreactive keratin (Fig. 3B) and actin (Fig. 3C) were abundantly present in the cytoplasm of the outer layer of cells that surrounded the ducts. Such basally situated cells were elongated in shape with their longitudinal axes running parallel to the duct. The outer peripheral cells lying around the swollen end buds, indented end buds and small clusters of buds (Fig. 3D) showed a variation in the intensity of staining with these two antisera. Cells at the tips of the swollen end buds often showed little or no staining, although occasional cells within the cortex were weakly stained. Where such buds were more septate, the cells within the clefts stained more intensely than the rest of the peripheral cells (not shown).

The basement membrane proteins laminin (Fig. 3E) and type IV collagen (not shown) showed a diffuse but continuous distribution around the parenchyma of the mammary implant. Basal cells, especially those lying around the end buds showed cytoplasmic staining (not shown). Fibronectin (Fig. 3F) and type I collagen (not shown) were located in the connective tissue surrounding the out-growths in a fibrous distribution.

Electron microscopy

Although the use of a whole-mount technique for locating the regenerated mammary gland within the interscapular fat pad was successful in selecting specific

Fig. 3. Histological sections. $\times 236$.

(A) An end bud in a mammary gland regenerated from implanted ducts, fixed after 6 weeks' growth, stained for MFGM. MFGM is present (arrows) on the luminal cells of the end bud (*eb*), duct (*d*) and small lateral bud (*lb*) and also shows some weak cytoplasmic staining in these cells.

(B) Section showing the same end bud as in A in which keratin is located within the peripheral cells (arrow) of the duct (*d*) and the end bud, although a few peripheral cells stain less intensely. Single cells within the cortex of the end bud also stain with anti-keratin serum (arrowhead). Note small lumina within the end buds (*l*) and loosely packed peripheral cells (arrowheads).

(C) A regenerated duct stained with antisera to actin shows a continuous layer of staining (arrow) within the cytoplasm of the elongated basal cells surrounding the duct.

(D) Alveolar buds stained with antisera to actin which stains 50–70 % of the basal cells.

(E) Ducts and end buds stained with antisera to laminin; a layer of laminin delineates (arrows) the ducts at the end of the end bud (*eb*), and blood vessels (*bv*) in the stroma.

(F) Fibronectin is located within the connective tissue sheath surrounding the duct (arrow) and is absent from the tip of the end bud (arrowhead).

areas for ultrastructural examination, preservation of the cell membranes was not as good as that achieved in normal glands using our routine fixation procedures (Ormerod & Rudland, 1984). These procedures include fixation with osmium tetroxide prior to dehydrating the tissue with graded alcohols. Nevertheless, sections from whole mounts still provided more detail than could be seen in the corresponding histological sections.

Different mammary cell types could be identified within the outgrowths, and these were the same for implants from bud-free ducts or TEBs. Polarized epithelial cells displaying apical microvilli lined the luminal surfaces of the ducts (Fig. 4A). Occasional apical-lateral junctions between adjacent luminal cells could be distinguished (Fig. 4A). The basal cells surrounding the ducts contained microfilaments, 5–8 nm in diameter, with focal dense areas within their cytoplasm (Fig. 4B). Hemidesmosomal attachments to the basement membrane lying along the basal surface of the cells were identified in some cells (Fig. 4B). The basal surface of these cells was often undulating with cytoplasmic projections, and pinocytotic vesicles were also present (Fig. 4B). The nuclei of basal cells showed a spectrum of morphologies; whereas some were indented and contained large amounts of peripheral heterochromatin, others had a smooth outline and little heterochromatin (Fig. 4B). Desmosomal attachments between the basal/myoepithelial cells and epithelial cells were also present (not shown). The ductal lumina sometimes appeared empty or contained an amorphous, granular material or cellular debris (Fig. 4A).

In the regenerated end buds, cells lying around their tips were cuboidal or irregular in shape and were on the whole more loosely packed than those within the central areas (Fig. 3A,B). These cells showed none of the characteristic features of myoepithelial cells seen in basal cells surrounding the ductal outgrowths (Fig. 5A). However, basal cells situated towards the neck of the end bud showed some peripheral heterochromatin within the nuclei, occasional small bundles of microfilaments containing one or two focal dense areas and hemidesmosome attachments to the basement membrane (Fig. 5B). The epithelial cells lying within the cortex of the TEB showed a spectrum of different morphologies (Fig. 5C). A few of the cells had a large pale cytoplasm and a smooth, round nucleus. Other epithelial cells which were often situated near the lumina had a dark cytoplasm and sometimes appeared necrotic. These cells were more irregular or columnar in shape and intersected the paler cells. The majority of the epithelial cells was closely packed and displayed a few microvilli where they lined lumina (Fig. 5C). The amount of connective tissue surrounding the end buds varied from a very narrow band of collagen fibres and elongated fibroblasts at the tips to a thicker sheath of connective tissue containing collagen, fibroblasts and small blood capillaries in the neck regions.

Both lateral buds and clusters of small buds were composed of epithelial cells lining the lumen (Fig. 6A) which were sometimes surrounded by a further layer of epithelial cells, and an outer layer of basal cells which were situated on the basement membrane (Fig. 6B). Their epithelial designation was confirmed by the

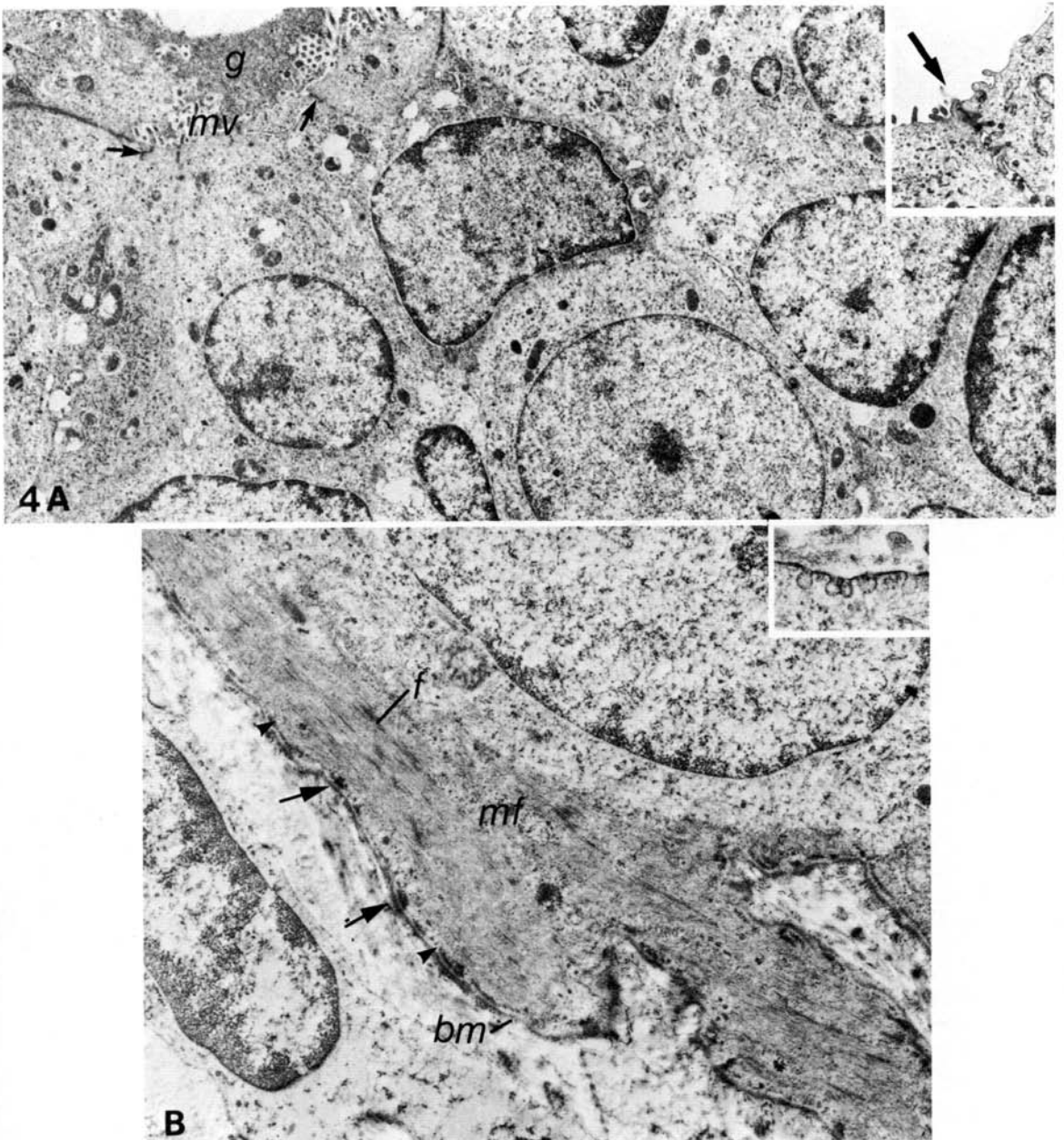


Fig. 4. Electron micrographs.

(A) Polarized epithelial cells that line the ducts. Note the microvilli (*mv*) on the apical surfaces of the cells, specialized junctions between the cells (arrows) and the presence of a granular material (*g*) within the lumen. $\times 7500$. Inset shows a junctional complex. $\times 12\,000$.

(B) A portion of cell showing some features of myoepithelial cells within a regenerated mammary duct after 8 weeks of growth. Microfilaments (*mf*) containing focal dense areas (*f*), pinocytotic vesicles (arrowheads) and hemidesmosomal attachments (arrows) to basement membrane (*bm*) are seen. $\times 13\,500$. Inset shows a higher magnification of pinocytotic vesicles. $\times 17\,000$.

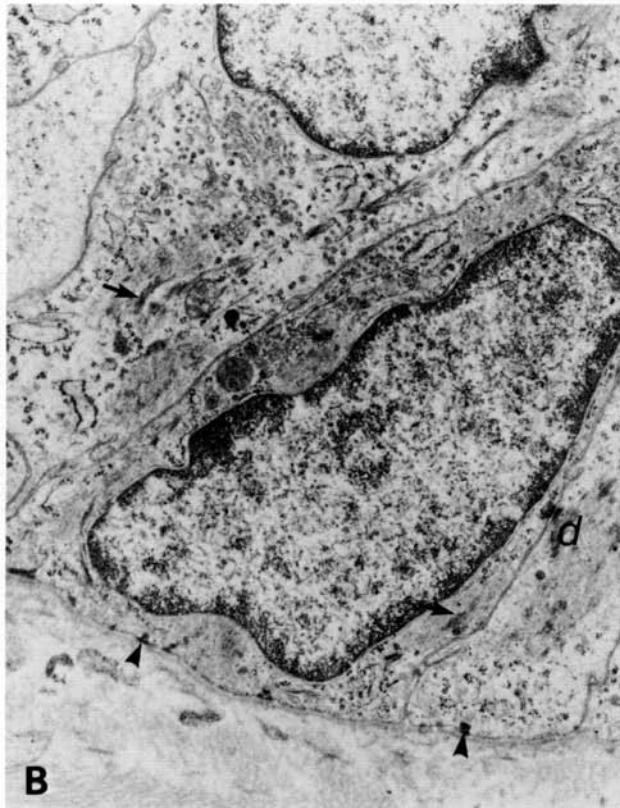
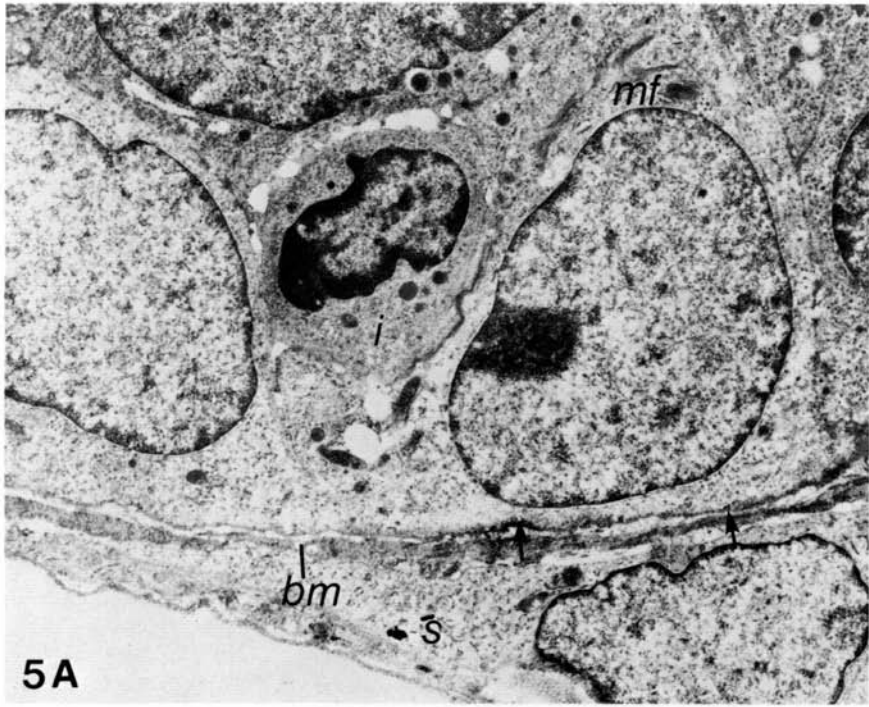


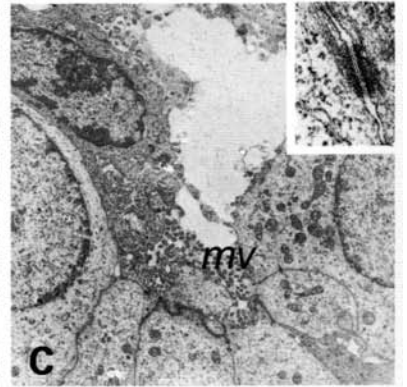
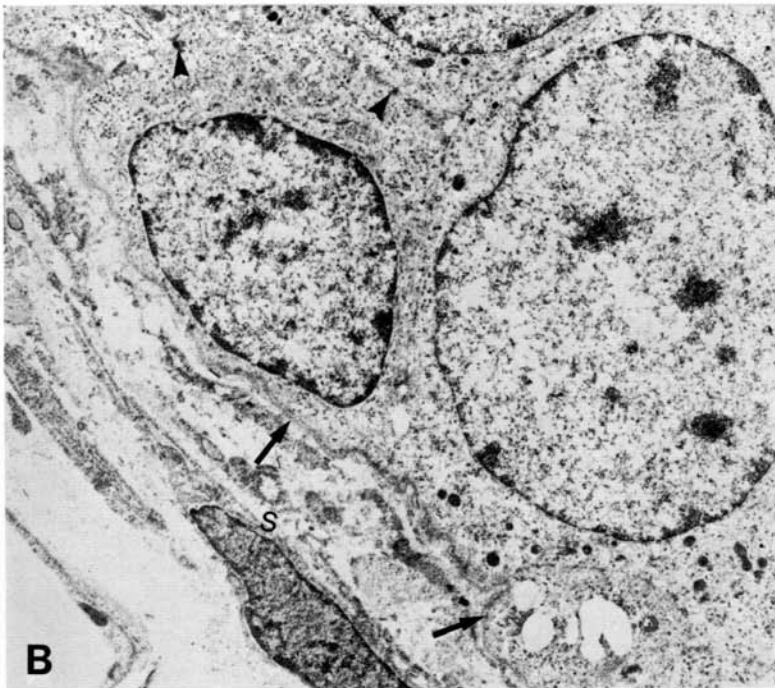
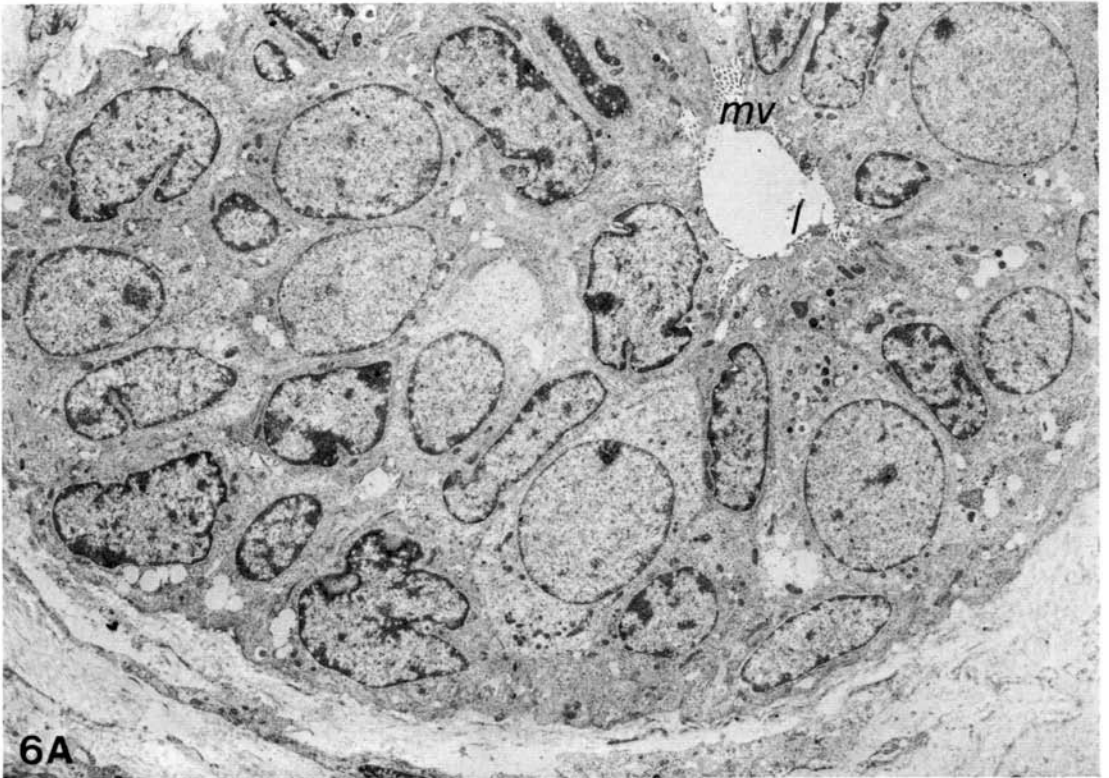


Fig. 5. Electron micrographs.

(A) Peripheral cells of an end bud. Scattered bundles of microfilaments (*mf*) are present in the apical cytoplasm of one cell, but no attachments to the basement membrane (*bm*) are present. A stromal cell (*s*) is in close proximity. Pinocytotic vesicles (arrows) are present along the basal membrane. Intraepithelial wandering cell (*i*). $\times 3300$.

(B) A basal cell situated at the neck of an end bud and containing microfilaments which contain some focal dense areas (arrows), desmosomes (*d*) joining adjacent cells, hemidesmosomes (arrowheads) attaching the cells to the basement membrane. $\times 13\,500$.

(C) A portion of an end bud composed of cells showing a variety of different nuclear morphologies. Cells lining the lumen have microvilli (*mv*) and junctional complexes (arrows). A basement membrane (*bm*) separates the basal undifferentiated cells (*b*) from the host stroma (*s*). $\times 5850$.



presence of microvilli (Fig. 6C) and junctional complexes. As seen in the end buds, some epithelial cells possessed round, smooth nuclei and their cytoplasm appeared paler than that of the more columnar epithelial cells seen within the buds. The columnar cells frequently converged on the lumina and resembled the basal cells of the tips of the buds in their nuclear and cytoplasmic morphology. However, they also formed desmosomal junctions and junctional complexes typical of epithelial cells. In 50–60 % of peripheral cells microfilaments could not be identified, in the remainder small bundles of filaments measuring 5–9 nm in diameter were seen (not shown). None of these cells, however, showed the abundant myofilaments seen in the basal cells of the ductal outgrowths.

DISCUSSION

Segments of both bud-free ducts and TEBs derived from normal rat mammary glands can regenerate a branching system of ducts when grown in the interscapular fat pad of syngeneic host rat. The implanted cells are capable of both organizing and growing as normal ducts which show regular spacings and a competition for spaces amongst themselves.

By the use of immunocytochemical and electron microscopic identification of the different cellular types, we have shown that both implanted bud-free ducts and TEBs can regenerate glands which have the same distribution of epithelial cells, myoepithelial cells and basement membrane proteins as in the normal prepubertal rat mammary gland from which they are derived. Thus, bud-free ducts have the potential to generate clusters of buds which are identical to the alveolar buds of the mammary gland, as well as generating new lateral buds and terminal end buds. The presence of casein within some of the cells composing these alveolar buds suggests that secretory alveolar-like cells are also produced in the regenerated glands. Similarly, mammary glands of newborn mice have the potential to undergo lobulo-alveolar development and lactate when isografted into mid-pregnant mice (Hoshino, 1983).

In implants derived from mammary bud-free ducts, the undifferentiated cells in the buds may originate either from similar cells that are already present within the mammary ducts or, under the correct environmental conditions, from the ductal epithelial or myoepithelial cells. The undifferentiated cells have been reported within mammary ducts (Radnor, 1971); however, it seems unlikely that they are

Fig. 6. Electron micrographs.

(A) A lateral bud composed of epithelial cells surrounding a lumen (*l*), lined in some parts by microvilli (*mv*). Note that cells within the bud display a variety of different nuclear morphologies: large cells with round nuclei and darker cells which have heterochromatic and indented nuclei. $\times 3000$.

(B) Higher magnification showing the basement membrane (arrows) which surrounds the lateral bud. Desmosomes (arrowheads) are present between adjacent cells. *s*, stromal cell. $\times 9900$.

(C) Higher magnification showing the microvilli (*mv*) present on the luminal surfaces of the cells. $\times 4300$. Inset shows a higher magnification of a desmosome junction between two adjacent cells. $\times 50\,000$.

present in sufficient quantities to produce the number of lateral buds along the ducts that are seen in the present study. Thus, it is more likely that, when the epithelial and, or, myoepithelial cells of the ductal termini are stimulated to divide, they form a bud of cells, some of which become dedifferentiated and act as a stem cell population for the cells within the newly formed outgrowth. However, unless sorted populations of cells of a defined phenotype are used for the implants, it is impossible to distinguish categorically between these two possibilities.

The fact that TEBs can regenerate many of the cells found in the mammary gland is not surprising in view of their suggested role in mammary development (Russo & Russo, 1978). Thus, in TEBs that lack fully differentiated myoepithelial cells, it seems likely that the undifferentiated cap cells at their tips convert into the myoepithelial cells of the subtending duct (Williams & Daniel, 1983; Ormerod & Rudland, 1984), and hence may be responsible for production of myoepithelial cells in the implants. Certainly, cells morphologically similar to the cap cells have been identified in the end buds of regenerated glands, and such cells also show gradations towards a myoepithelial phenotype. The epithelial cells in the implants may either arise from the epithelial cells themselves or from the cap cells of the TEBs, since there is some evidence that the cap cells may also be responsible for the production of epithelial cells within the TEBs *in vivo* (Williams & Daniel, 1983). It is probable that the myoepithelial cells are responsible for much of the synthesis of the basement membrane, since cytoplasmic staining of the basal, myoepithelial cells with antisera to basement membrane proteins was observed, in agreement with earlier studies *in vivo* (Wicha, Liotta, Vonderhaar & Kidwell, 1980; Warburton *et al.* 1982). On the other hand the connective tissue sheath around the regenerated ducts probably arises from cells within the surrounding host adipose tissue, although the possibility that the few adherent stromal cells present on the transplanted segments (not shown) could have contributed cannot be ruled out.

In conclusion, both transplanted bud-free ductal segments and TEBs from normal glands can regenerate fully developed mammary glands that show histological features identical to those of the host. This demonstrates that the regenerative and differentiating ability of the mammary gland is not confined solely to cells within its budded regions, and suggests that normal ductal epithelial/myoepithelial cells also possess this ability, but it is only manifested in the appropriate environment.

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