Cytodifferentiation of mouse mammary epithelial cells cultured on a reconstituted basement membrane reveals striking similarities to development *in vivo*

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Summary

In the present study we provide evidence that the cytodifferentiation of primary mouse mammary epithelial cells within the alveolar-like structures formed after culture on a reconstituted basement membrane resembles development *in vivo* during late pregnancy and early lactation. During the first two days in culture on a basement membrane gel in the presence of lactogenic hormones, epithelial cells isolated from mid-pregnant mice are disorganized and central lumina are largely absent. Levels of mRNA for the milk proteins, β-casein and transferrin, are dramatically reduced. By the second or third day in culture, cytoplasmic polarization becomes evident and prominent apical junctional complexes are formed. Synthesis of both mRNA and milk protein is reinitiated at this time. By day 4, well-defined lumina appear, and abundant synthesis and secretion of casein and lipid is observed. A striking feature of this differentiation in culture is the specific localization of milk protein gene expression (β-casein mRNA) to luminal epithelial cells in the alveolar-like structures. At the ultrastructural level, increased milk protein synthesis and secretion are paralleled by a fourfold increase in rough ER that resembles the dramatic increase in the ER observed *in vivo* following parturition. One indication of tissue-specific differentiation observed in later cultures (days 4–11) is the synthesis and secretion of abundant casein micelles. A second characteristic of lactating mammary epithelial cells *in vivo* that has not previously been observed in culture is the secretion of milk fat globules. Taken together, these observations indicate that mammary epithelial cells plated onto a reconstituted basement membrane differentiate to the lactating phenotype in culture.

Key words: *in situ* hybridization, casein micelles, milk fat globule, β-casein, basement membrane.

Introduction

The development and function of the mammary gland have been subjects of intense interest for many years. Pioneering studies of its morphological differentiation during pregnancy and lactation were among the earliest electron-microscopic investigations to trace the subcellular route of newly synthesized secretory proteins from the rough endoplasmic reticulum (RER) to the *trans* Golgi face, where secretory vesicles are formed (Wellings *et al.* 1960; Bargmann, 1962; Kurosumi *et al.* 1968; Wellings, 1969). During the past decade, many studies have been focussed on both molecular and cellular aspects of milk protein gene expression. A variety of milk protein genes have now been cloned, and their regulatory sequences are beginning to be dissected (Bisbee and Rosen, 1987). At the same time the cellular mechanisms underlying the expression of milk proteins have been studied in some detail, especially through the development of mammary epithelial cell culture systems (Bissell and Hall, 1987). These cell culture models confer many advantages for investigating the mechanisms underlying differentiation and tissue-specific gene expression, including the ability to isolate a single cell type, to manipulate hormonal stimuli and other growth conditions, and to control the microenvironment, including cell–cell and cell–matrix interactions. In the case of the mammary epithelium, such studies have made it clear that the underlying basal lamina and its components exert important control over milk protein expression (Li *et al.* 1987; Aggeler *et al.* 1988; Streuli and Bissell, 1990).

Our previous work has indicated that mammary epithelial cells derived from mid-pregnant animals and cultured on a reconstituted basement membrane re-form alveolar-like structures and synthesize and secrete milk proteins into sealed lumina (Barcellos-Hoff *et al.* 1989). We now report that alveolar re-formation is accompanied by differentiation of these cultured mammary epithelial cells from a mid-pregnant phenotype to one closely resembling lactation. In addition, expression of the tissue-specific milk protein, β-casein, appears to be localized to highly differentiated luminal epithelial cells.

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Materials and methods

Cell culture

Primary mouse mammary epithelial cells were isolated from mid-pregnant (13- to 15-day) CD-1 mice (Charles Rivers Laboratories, Wilmington, MA), as previously described (Lee *et al.* 1984; Bissell *et al.* 1987; Barcellos-Hoff *et al.* 1989). After mincing and digestion with collagenase and trypsin, epithelial cells were separated by differential low-speed centrifugation, resuspended in F-12 medium containing 10 % fetal bovine serum (both from Gibco, Grand Island, NY), and plated at 2×10^5 to 5×10^5 cells cm^{-2} in culture dishes precoated with a reconstituted basement membrane extract isolated from the Englebreth-Holm-Swarm (EHS) tumor (Kleinman *et al.* 1986). Serum was removed after 2 days of culture and cells were maintained in serum-free medium for the remainder of the experiment. Fresh medium was added every day. All cultures were carried out in the presence of the lactogenic hormones, ovine prolactin ($3\,\mu g\,ml^{-1}$; National Hormone and Pituitary Program, National Institutes of Health, Bethesda, MD), hydrocortisone ($2\,\mu g\,ml^{-1}$) and insulin ($5\,\mu g\,ml^{-1}$) (both from Sigma Chemical Co., St Louis, MO), which were added daily. The isolated cell preparations contained >90 % epithelial cells (Seely and Aggeler, 1991), as judged by staining for cytokeratins (Lane, 1982).

Immunofluorescence microscopy and in situ *hybridization*

Pregnant and lactating mammary gland and cultured mammary epithelial cells were prepared for immunofluorescence microscopy or *in situ* hybridization by fixation with 2 % paraformaldehyde, followed by freezing and sectioning with a Leitz cryotome, as previously described (Streuli and Bissell, 1990). Immunofluorescence microscopy was carried out using a β-casein-specific mouse monoclonal antibody (kindly supplied by C. Kaetzel, Case Western Reserve University, Cleveland, OH), followed by biotinylated rabbit anti-mouse IgG and streptavidin–Texas Red (both from Amersham Corp., Arlington Heights, IL). Slides were counterstained with 4′,6′-diamidino-2-phenyl-indole (DAPI) (Sigma) to localize cell nuclei. The distribution of β-casein mRNA was determined by *in situ* hybridization, according to the method of Cox *et al.* (1984). A total of 540 bases of mouse β-casein cDNA coding sequence (originally isolated by Dr Jeffrey Rosen, Baylor College of Medicine, Houston, TX) were recloned into transcription vectors (PGEM-1, Promega Corp., Madison, WI; and $pT_7/T_3\alpha19$, Bethesda Research Labs, Gaithersburg, MD) and high specific activity ^{35}S-labeled riboprobes (sense and antisense, respectively) were prepared by transcription using bacteriophage T7 RNA polymerase. Frozen sections were applied to organosialinized microscope slides (Turtellotte *et al.* 1987), treated with proteinase K ($0.1\,\mu g\,ml^{-1}$), postfixed with 2 % paraformaldehyde, acetylated and dehydrated through a series of graded ethanols (Martins-Green and Bissell, 1990). Slides were typically hybridized with 10^6 cts min^{-1} of labeled probe overnight at 55 °C, washed, treated with RNAase A ($20\,\mu g\,ml^{-1}$) in 2×SSC for 30 min, washed again, coated with Kodak NBT-2 photographic emulsion, and exposed at −20 °C for 2–5 days before development and staining with Diff-Quick.

Determination of milk protein mRNA

Purification of mRNA and Northern blot analysis were carried out as previously described (Streuli and Bissell, 1990). Gels were run with $2\,\mu g$/lane total RNA and loading was judged to be equal, on the basis of ethidium bromide staining of the 18 S and 28 S RNA bands after electrophoresis.

Transmission electron microscopy

Cells grown in 16-mm diameter wells coated with EHS matrix were fixed with 2 % glutaraldehyde in Hanks' balanced salt solution containing 20 mM Hepes (pH 6.8) for 1 h at room temperature, followed by postfixation with 1 % OsO_4 for 1 h at 4 °C. Fixed cells were dehydrated and embedded in Epon/Araldite in the culture dish, then re-embedded and sectioned perpendicular to the dish, as previously described (Li *et al.* 1987). Ultrathin sections were stained with lead citrate and examined in a Philips 400 electron microscope at an accelerating voltage of 80 kV. In one experiment, cells were postfixed with 1 % tannic acid for 1 h at room temperature before osmication.

Morphometry

To quantify lumen formation, cells cultured on EHS matrix for 1–8 days were fixed for 1 h with 2 % paraformaldehyde in phosphate-buffered saline (pH 7.0), washed and embedded in paraffin. Thick sections (5 μm) were cut, mounted on microscope slides, and stained with hematoxylin–eosin. Relative cross-sectional areas occupied by cells and lumina were determined by point counting at the microscope using a ×40 Neofluar objective and a multipurpose test grid with 42 points, as described by Hyde *et al.* (1990). At least 480 points falling on cells or lumina were counted for each slide (duplicate slides for each time point). To quantify lipid droplets in cells and lumina, the same slides were recounted with a ×63 objective (at least 990 points per slide). To determine the size distribution of casein micelles, 63 electron micrographs (original magnification ×27 500) were taken, covering the entire cross-sectional surface of a typical alveolar profile in a 4-day culture. These micrographs were printed at a final magnification of ×65 000 and the diameter of every micelle was measured to the nearest mm using a magnifying graticule. A total of 1802 micelles were measured (159 intracellular and 1643 in the lumen). To monitor the development of the protein synthetic apparatus during alveolar differentiation, electron micrographs (original magnification ×4600) were taken, covering the entire cross-sectional surface of at least 4 structures/day in culture (2–10 micrographs/structure). These micrographs were printed at a final magnification of ×12 500 and scored for the presence of cytoplasmic structures by point counting using an isotropic test grid (Hyde *et al.* 1990). Volume/surface ratios ($\mu m^3/\mu m^2$) for RER, Golgi apparatus, and lipid droplets were calculated using the formula:

$$\frac{V\,(\mu m^3)}{S\,(\mu m^2)} = \frac{L_T}{2I_{BL}} = \frac{\pi D P_R}{2} \times \frac{1}{2I_{BL}} = \frac{0.7854 D P_R}{I_{BL}},$$

where L_T is test line length, D is distance between points on the test grid=1.52 cm; P_R, points falling on the reference structure; and I_{BL}, line intersections with the basal lamina.

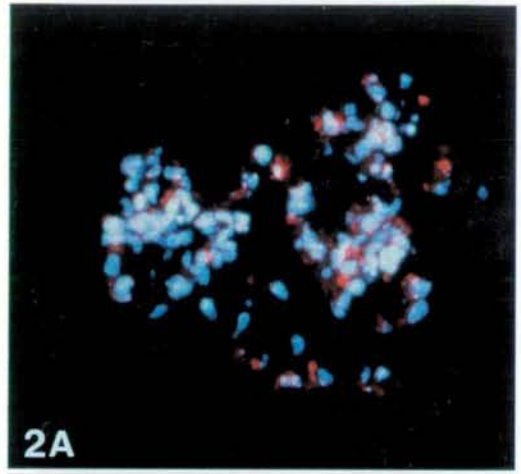
Results

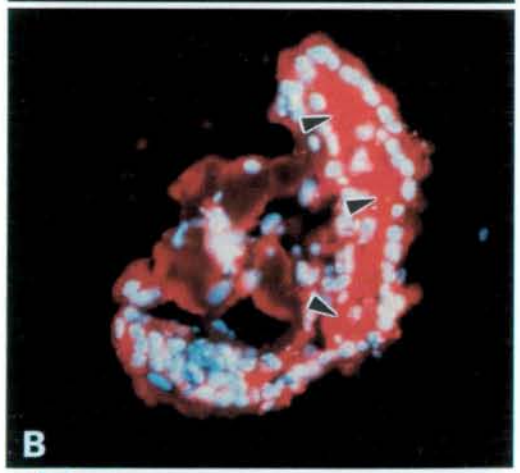
Growth of lumina in culture

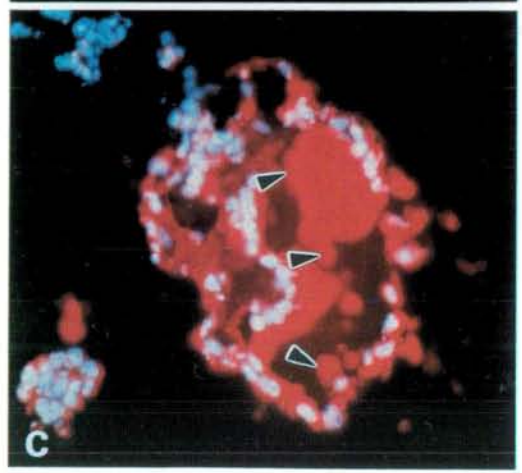
Epithelial cells are isolated from mid-pregnant mouse mammary gland as a mixture of single cells and small clusters of 10–40 cells, which attach to the EHS matrix and appear to pull it up around them, so that they come to be completely surrounded by this basement membrane material (Barcellos-Hoff *et al.* 1989; Seely and Aggeler, 1991). For the first few days in culture, lumina are either collapsed or absent (Figs 1, 2). By day 4, apical/basal polarity has been re-established and lumina are found in cell aggregates throughout the cultures. Synthesis and vectorial secretion of milk proteins are observed at this time, as seen in sections of cultures stained with a monoclonal antibody against β-casein (Fig. 2B,C). Accumulation of newly synthesized milk proteins in lumina correlates with a twofold increase in lumen size between days 4 and 8 in culture (Fig. 1).

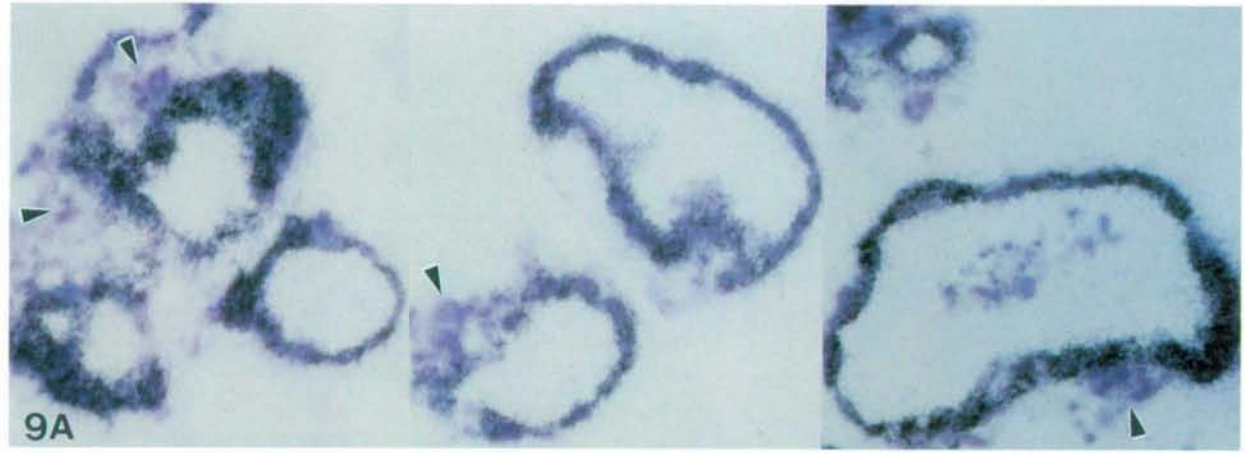
Cytoplasmic differentiation of cells from pregnant to lactating phenotype

Cells of the mid-pregnant mammary gland are characterized by a relatively high nuclear to cytoplasmic ratio, isolated ER cisternae, well-developed Golgi apparatus and limited ability to synthesize casein, as evidenced by some casein micelle formation (Fig. 3A). After parturition, the









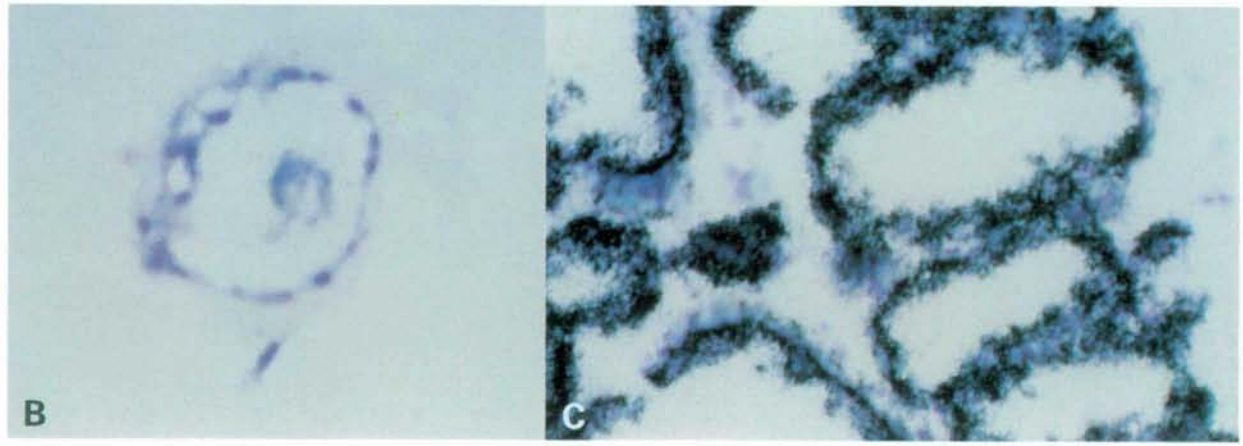


Fig. 9. Localization of β-casein mRNA in epithelial cells cultured on EHS matrix. Cells from 6-day cultures (A,B) or from lactating gland (C) were processed for *in situ* hybridization using ^{35}S-labeled antisense (A,C) or sense (B) probes for β-casein mRNA. In cells from EHS cultures (A) or lactating gland (C), β-casein mRNA is exclusively localized within alveolar epithelial cells. Cells that have not been incorporated into alveoli in culture do not appear to express this gene (A) (arrowheads). Very few background silver grains are observed in sections hybridized with a sense riboprobe (B). ×280.

Fig. 2. Synthesis and secretion of β-casein into lumina in EHS cultures. Cells cultured on EHS matrix for 1 (A), 4 (B) or 6 (C) days were incubated with an antibody against β-casein, followed by staining with a biotinylated secondary antibody and streptavidin–Texas Red. Little intracellular milk protein is observed at early times in culture (A), but accumulation of abundant β-casein in swollen lumina (arrowheads) is obvious in cultures on days 4 (B) and 6 (C). Sections have been counterstained with DAPI to localize cell nuclei, which fluoresce blue or white. ×280.

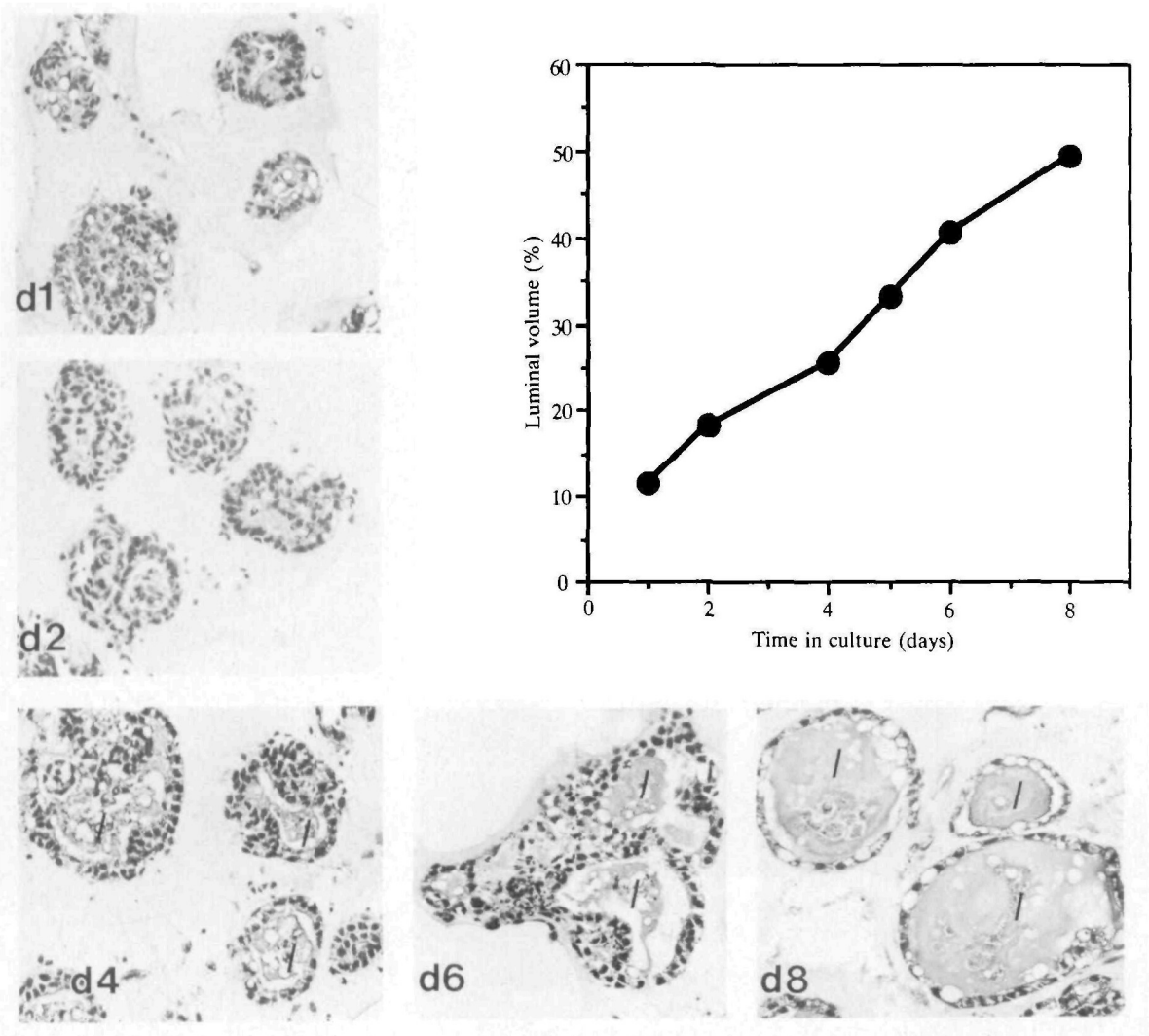


Fig. 1. Development of lumina on EHS matrix. Epithelial cells were cultured on EHS matrix for 1, 2, 4, 5, 6 or 8 days. By day 4, the cells have become polarized and begin synthesizing and secreting milk proteins into the lumina (*l*), which subsequently swell as they become engorged with secreted milk. Morphometric analysis demonstrates that the volume occupied by lumina increases approximately 5-fold between days 1 and 8 of culture. ×2000.

synthetic capacity of mammary epithelium is increased markedly, and cells become filled with endoplasmic reticulum, swollen Golgi profiles and secretory vesicles (Fig. 3C). At the same time, milk fat globule secretion is observed. Cells from mid-pregnant mice cultured on the EHS matrix undergo a similar transition from a phenotype characteristic of the pregnant gland on day 4 (Fig. 3B) to one reminiscent of lactating gland from day 6 on (Fig. 3D).

The abundant synthesis and secretion of milk proteins (Fig. 2) suggest that mammary epithelial cells maintained on EHS matrix differentiate from a pregnant to a lactating phenotype in culture. Such differentiation is also indicated by the dramatic increase in the amount of RER during culture (Fig. 4A). In well-differentiated cultures, the hypertrophied RER is present as prominent arrays of ribosome-studded membrane stacks found throughout the cytoplasm (Fig. 4B). In a small proportion of cells (<5%), the RER appears swollen, and active formation of casein-containing secretory vesicles is not evident (Fig. 4B). Hurley *et al.* (1989) recently reported that similar swollen RER cisternae in mammary epithelial cells cultured on floating type I collagen gels contain β-casein, and suggested that the swelling was caused by a block in the secretory protein sorting pathway. That this pathway is intact and functioning normally in EHS cultures is confirmed by the observation of abundant casein micelle formation in the *trans* Golgi network of these actively synthesizing cells (Fig. 4D; also see Fig. 5C). As shown in Fig. 4A, the differentiation that we observe from the pregnant phenotype to the actively secretory lactating one is not accompanied by an increase in Golgi profiles in these cells. Rather, as shown in Fig. 4C, Golgi stacks in early, non-synthetic cultures appear well-organized, but simply lack any indication of the casein micelle formation characteristic of the fully differentiated later cultures. This is also consistent with the low levels of casein synthesis and secretion observed during the first 2–3 days in culture (Fig. 2; Barcellos-Hoff *et al.* 1989).

Milk-specific secretion in culture

The caseins are a group of phosphoproteins that are secreted in crystalline form as micelles during lactation. One of the most striking characteristics of mouse

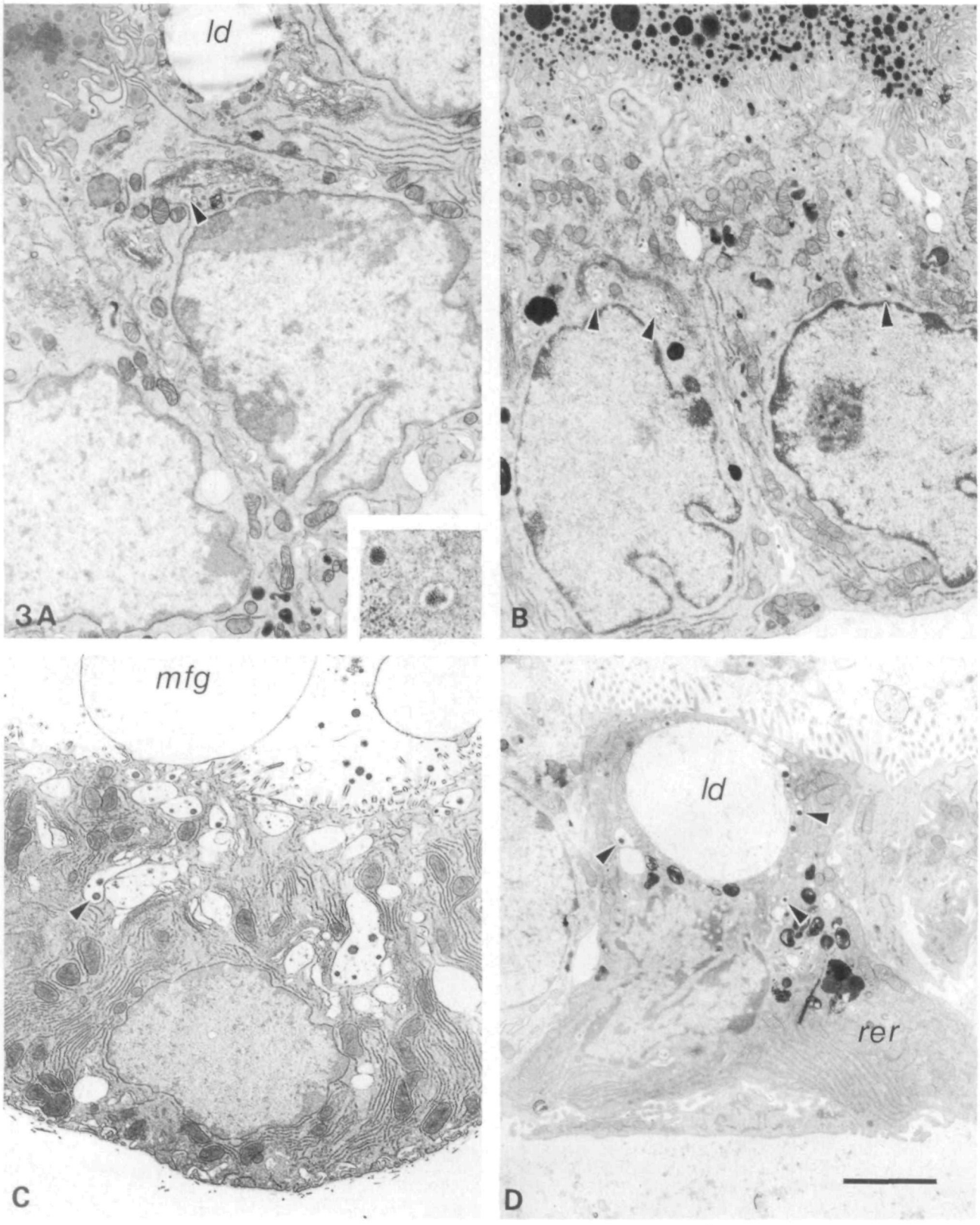


Fig. 3. Differentiation of cells from mid-pregnant mammary gland to a lactating phenotype in culture. Mammary gland from mid-pregnant (A,B,D) or lactating (C) mice were fixed immediately (A,C) or dissociated and cultured on EHS matrix for 4 (B) or 6 (D) days before being processed for electron microscopy. The actively synthetic lactating phenotype (C,D) shows abundant stacks of rough endoplasmic reticulum (*rer*) and synthesis and secretion of casein micelles within swollen secretory vesicles (arrowheads) (A, inset). Secreted lipid in the form of milk fat globules (*mfg*) is a prominent feature of the lactating phenotype (C). Intracellular lipid droplets (*ld*) are present in both pregnant (A) and lactating cells (D). Bar, 2 μm. ×8000. Inset: ×40 000.

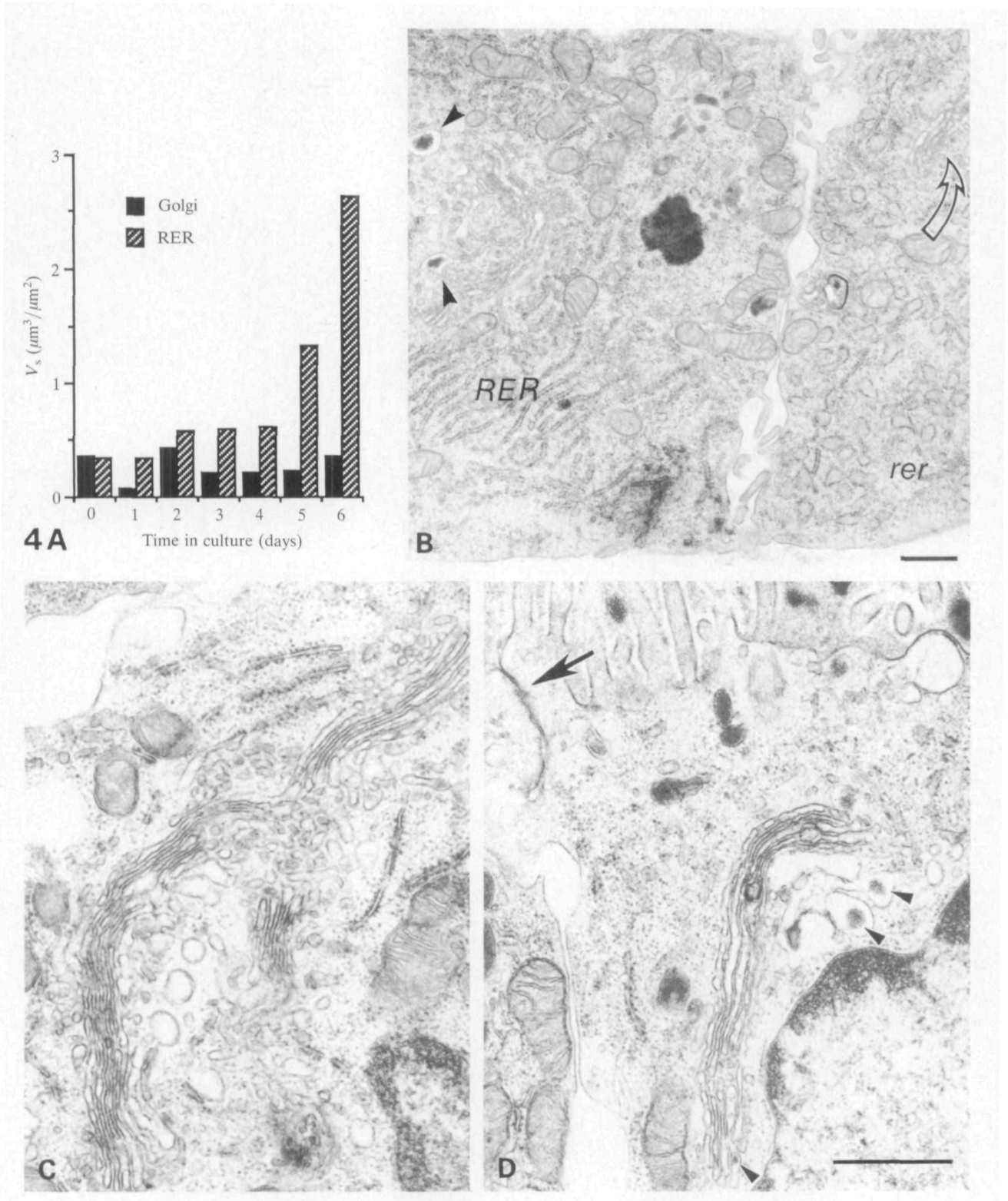


Fig. 4. Development of protein synthetic organelles in epithelial cells cultured on EHS matrix. To quantify organelle development, representative sections of cells from 1- to 6-day cultures were scored for the presence of RER (hatched bars) and Golgi apparatus (solid bars), and the ratios of organelle volume to basal laminar surface area were determined (A). In cells cultured for 6 days on EHS matrix (B), the abundant stacks of RER typical of lactating cells are evident. Arrowheads indicate casein micelles forming at the *trans* face of the Golgi apparatus. Occasional cells in these cultures show swollen endoplasmic reticulum (*rer*), as observed in the cell on the right (B). Open arrow indicates an apparently inactive Golgi profile, which lacks clear evidence of casein micelle formation in this cell. During the early days of culture, the Golgi apparatus of isolated mammary epithelial cells remains intact, but shows little indication of active transport of newly synthesized protein (C, day 2). After lumina sealed by apical junctions (arrow) form, casein micelles are frequently observed forming at the *trans* face of the Golgi (arrowheads), as seen on day 4 in (D) and day 6 in (B). Bar, 0.5 μm. B, ×19 000; C and D, ×39 000.

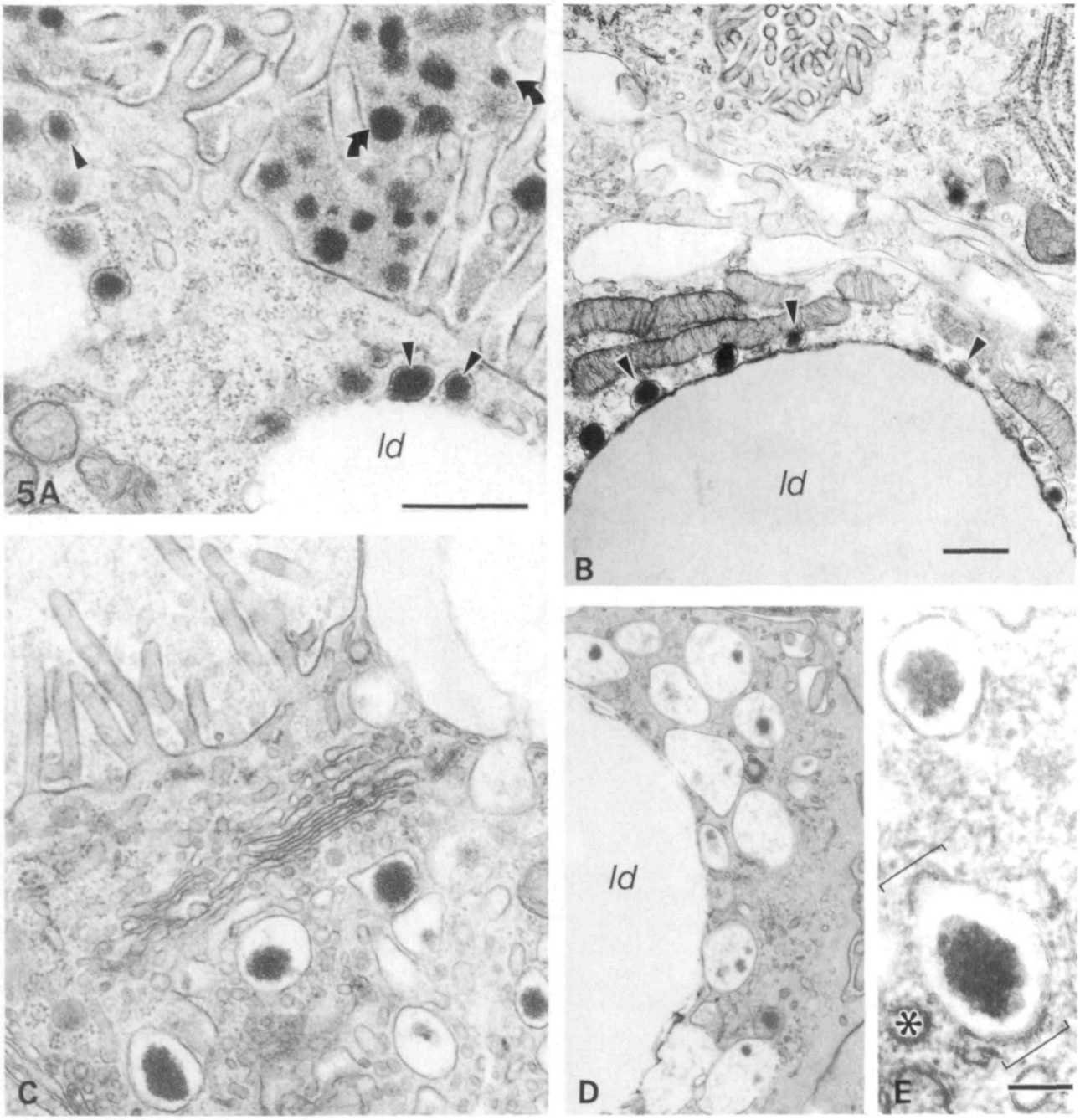


Fig. 5. Casein micelle formation by cultured mammary epithelial cells. In cells cultured on EHS matrix for 4 (A,D), 6 (B,C) or 8 days (E), both intracellular (arrowheads) and secreted (curved arrows) micelles are abundant. Single (A) and multiple micelles (D) are observed in secretory vesicles in the apical cytoplasm, at the *trans* Golgi face (C), and in association with intracellular lipid droplets (*ld*) (A,B). In an 8-day culture that was postfixed with tannic acid (E), areas of clathrin basket formation (brackets) are observed on a casein-containing secretory vesicle; asterisk indicates a small coated vesicle. Bar, A–D, 0.5 μm; E, 0.1 μm. A and C, ×40 000; B and D, ×20 000; E, ×100 000.

mammary epithelial cells cultured on EHS matrix is the formation and secretion of abundant casein micelles (Fig. 5A), a functional marker infrequently observed in cultured cells. After 4 days in culture secretory vesicles containing micelles are present in virtually all luminal cells in the apical cytoplasm (Fig. 5A), associated with intracellular lipid droplets (Fig. 5B), and at the *trans* face of the Golgi apparatus (Fig. 5C). In some synthetically active cells, multiple micelles are observed within a single secretory vesicle (Fig. 5D), as is also observed in lactating gland (Fig. 3C). As has been previously reported (Franke *et al.* 1976), small areas of clathrin coat were observed on some casein-containing secretory vesicles in a culture postfixed with tannic acid (Fig. 5E). Micelles secreted into the lumen in a day 4 culture had a modal diameter of approximately 80 nm and a size range of 15 to >300 nm (Fig. 6), similar to the values reported for micelles in lactating gland (Wellings *et al.* 1960; Hollmann, 1974). A smaller sample of intracellular micelles showed a similar distribution, but lacked the very large micelles observed

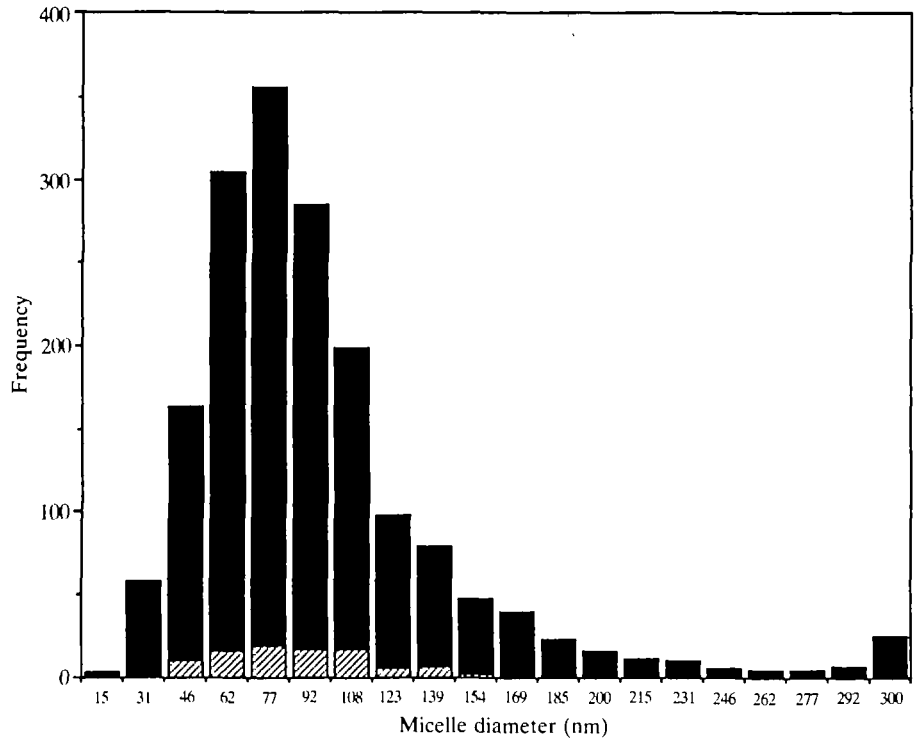


Fig. 6. Casein micelle size distribution. To determine the apparent size distribution of the casein micelles formed by cultured mammary epithelial cells, micelle diameters were measured on a set of electron micrographs that covered the cytoplasm and lumen of a representative mid-luminal section from a 4-day culture (see Fig. 2). Secreted micelles are shown in black (n=1643) and intracellular ones are crosshatched (n=159).

in the lumen, which presumably represent aggregates that form after secretion.

In addition to milk proteins, another important component of milk is fat, secreted as characteristic milk fat globules, which are extruded from mammary epithelial cells surrounded by a sheath of apical plasma membrane. Although fat synthesis and formation of intracellular lipid droplets are observed in mammary epithelial cells during pregnancy, extrusion of well-formed milk fat globules does not occur (Mather, 1987). In contrast, mammary epithelial cells cultured on EHS matrix for 6–8 days show evidence of milk fat globule formation (Fig. 7A) and extrusion into the lumen (Fig. 7B). Between days 2 and 8, total lipid in these cultures increases approximately 2.6-fold, while lipid droplets secreted into lumina increase by >5-fold (Table 1). Interestingly, intracellular lipid remains relatively abundant during the first 3 days in culture and then decreases dramatically on day 4, a pattern similar to that observed *in vivo* at parturition (Hollman, 1969, 1974). As is observed in the lactating mammary gland (Fig. 7C), milk fat globules formed in culture are extruded from the cell surrounded by a membrane derived from the apical plasma membrane (Fig. 7D).

Table 1. *Milk fat globule formation in culture*

Time in culture (days)	Total lipid* (%)	Luminal lipid* (%)	Intracellular lipid† ($\mu m^3/\mu m^2$)
1	–	–	1.07
2	7.1	1.5	2.15
3	–	–	1.42
4	9.1	3.2	0.41
5	–	–	0.33
6	12.2	5.5	3.23
8	18.6	7.6	–

*Volume densities of lipid were determined by point counting at the microscope, as described in Materials and methods, and are expressed as % of total points falling on alveolar structures in these cultures.

†Volume/surface ratios of intracellular lipid were determined from the same set of electron micrographs as shown in Fig. 5, and as described in Materials and methods.

Expression of milk protein genes in luminal epithelial cells

We have shown previously that mammary epithelial cells cultured on EHS matrix for a week or more contain high levels of mRNA for the milk proteins β-casein (Li *et al.* 1987) and transferrin (Chen and Bissell, 1987), compared to cells grown on plastic. When we measured mRNA for these proteins during early times in culture on EHS matrix, a striking decrease to almost undetectable levels was observed (Fig. 8, lane 1). By day 3, mRNAs for both proteins were again abundant, demonstrating re-expression of these tissue-specific genes. Using *in situ* hybridization with an antisense probe for β-casein mRNA, milk protein expression was clearly localized within epithelial cells lining the lumina in EHS cultures and was absent from non-luminal cells (Fig. 9A).

Discussion

Regulation of differentiation to lactation in culture

The mammary gland is a rich model system for studying the mechanisms that control tissue-specific gene expression, both because of the number of gene products available and because of the hormone-regulated cyclical nature of this expression. Nevertheless, as with many other complex biological processes, sorting out the relative contributions of a variety of regulatory mechanisms to the induction and modulation of milk protein synthesis has been difficult to accomplish *in vivo*. For this reason, considerable effort has been expended to develop cell culture system(s) in which these putative regulatory molecules can be tested directly. The present results highlight the power of basement membrane matrices not only to support milk protein gene expression in cultured mammary epithelial cells, but also to permit developmental progression that mimics events *in vivo*. We now have a cell culture model in which the signals regulating

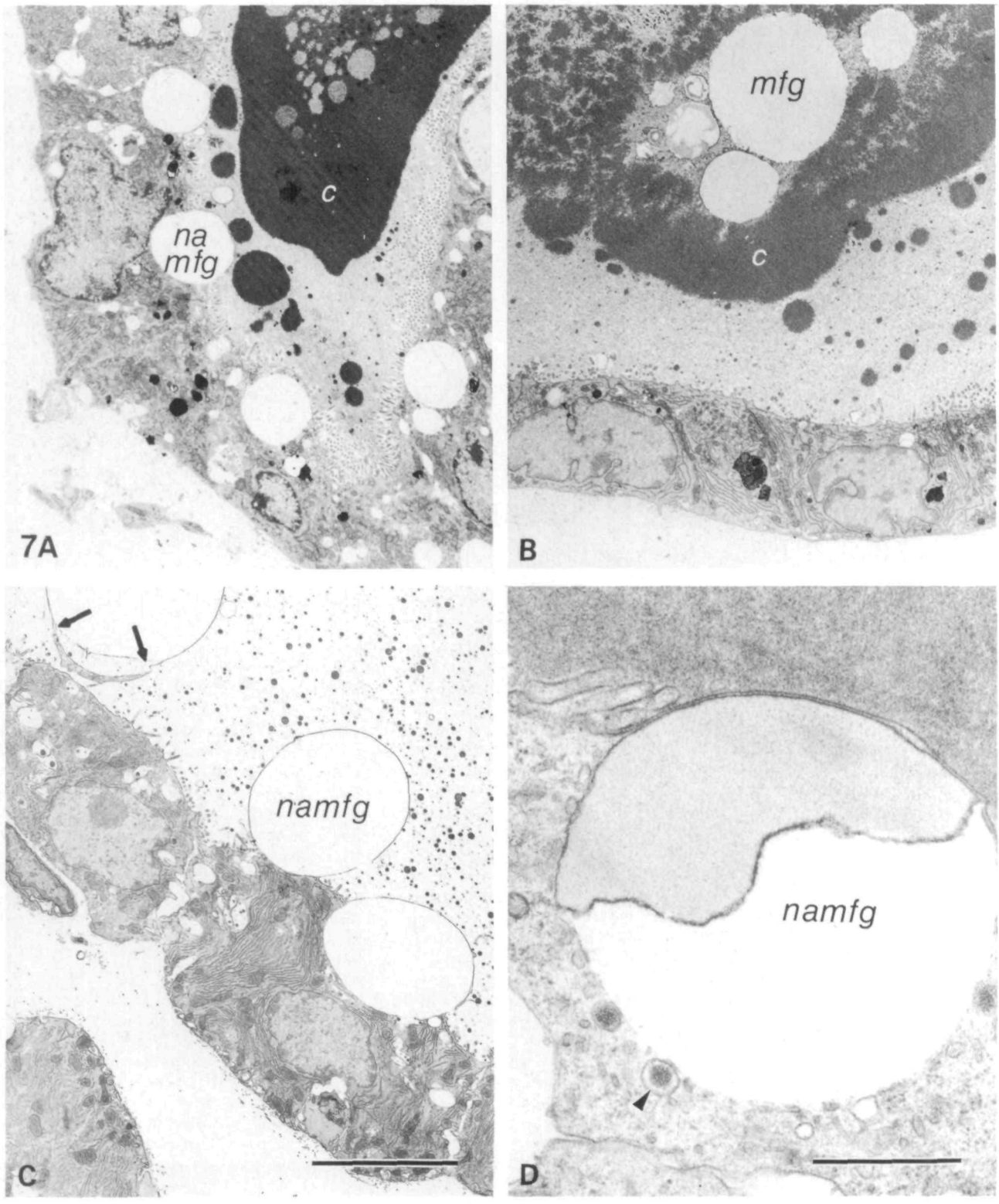


Fig. 7. Milk fat globule formation in culture. Epithelial cells cultured on EHS matrix for 6 (A,D) or 8 (B) days show evidence of abundant milk fat globule formation, similar to that observed in cells in lactating gland (C). Both budding from the apical cell surface (A) and the presence of membrane-bounded lipid in lumina (B) are observed in later cultures. Large aggregates of casein (*c*) are also found in the lumina in these cultures. Arrows in C indicate a mfg in lactating gland with which a small amount of apical cytoplasm has apparently been included. At high magnification (D), the close apposition of the apical plasma membrane to a nascent milk fat globule (*namfg*) in a 6 day culture is seen; arrowhead indicates small casein-containing secretory vesicles that are often observed at the cytoplasmic face of these lipid droplets. Extraction of the fatty contents of the cytoplasmic half of this droplet reflects difficulty in preserving lipids during fixation and processing for electron microscopy. Bars: A, B and C, 5 μm; D, 0.5 μm. A, B and C, ×4000; D, ×40 000.

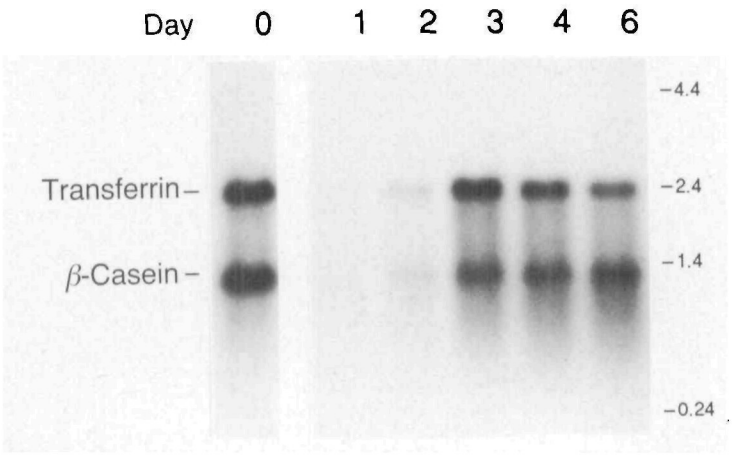


Fig. 8. Milk protein mRNA levels in cells cultured on EHS matrix. Total RNA was isolated from mammary epithelial cells at the time of plating (0) or after 1, 2, 3, 4 or 6 days in culture. Northern blot analysis indicates that β-casein and transferrin mRNAs fall to very low levels during the first day in culture. By day 3, both mRNAs have been resynthesized, and mRNA levels remain high at least through day 6 of culture. The positions of RNA-size markers (in kb) are noted in the margin.

differentiation from a pregnant to a lactating phenotype can be studied.

Hypertrophy of the secretory apparatus

The intracellular route followed by newly synthesized secretory proteins is well-established, and actively secretory cells, are usually characterized by abundant RER cisternae and well-developed Golgi stacks. Early morphometric studies by Hollman (1969, 1974) indicated that the RER develops during the first half of pregnancy and then remains essentially unchanged during the last week of gestation (approximately days 14 to 21 in the mouse). Beginning at parturition, there is a dramatic hypertrophy of the RER, which increases by threefold during the first 5 days of lactation. This pattern is closely paralleled by the extent of RER development that we observe in mammary epithelial cells cultured on EHS matrix (approximately fourfold increase between days 4 and 6), although the timing of these changes is somewhat accelerated compared to the situation *in vivo* (3–4 days in culture compared to a week *in vivo*). Nevertheless, the pattern and extent of RER development support the conclusion that epithelial cells cultured on EHS matrix differentiate from a pregnant to a lactating phenotype.

Formation of casein micelles

Alveolar epithelial cells of the lactating mammary gland synthesize a family of calcium-binding phosphoproteins, the caseins, which form characteristic crystalline aggregates or micelles within secretory vesicles at the *trans* Golgi face of actively secreting cells (Wellings *et al.* 1960; Hollmann, 1974). These micelles have a fairly broad size distribution, ranging from 40 to 300 nm in diameter in mouse mammary gland (Wellings *et al.* 1960) to 30 to 600 nm in diameter in bovine milk (McGann *et al.* 1980). Both the range (15 to 600 nm) and the distribution (modal diameter 80 nm) of casein micelles synthesized by cells cultured on EHS matrix are essentially identical to those found in lactating gland. The size of individual micelles is apparently determined by the proportion of κ-casein incorporated, with smaller micelles having more of this casein species than larger ones (McGann *et al.* 1979, 1980). This relationship suggests that, in addition to α- and β-casein (Barcellos-Hoff *et al.* 1989), κ-casein is also being synthesized in abundance by cells cultured on EHS matrix. Casein micelles have occasionally been seen in mammary epithelial cells in organ (Mills and Topper, 1970) or cell culture (Hamamoto *et al.* 1988; Hahm *et al.* 1990), but not in the numbers that we observe in cells cultured on EHS matrix. Although some micelles are formed as early as day 8 of pregnancy (Aggeler *et al.* 1990), significant secretion is not observed, and those micelles that do form during pregnancy may be degraded intracellularly, a form of milk stasis (Hollmann, 1974). Only after parturition do micelles accumulate in large numbers in the alveolar lumina, a condition that is paralleled by cells cultured on EHS matrix for four or more days. The presence of multiple micelles within individual swollen secretory vesicles reflects amounts of casein synthesis similar to those observed in lactating gland, and the marked accumulation of fluid in these vesicles may indicate increased lactose synthesis, as well.

Formation of milk fat globules

Milk contains fat as a readily available energy source. During pregnancy, some fat synthesis takes place and lipid droplets, sometimes quite large, are commonly found free in the cytoplasm of secretory epithelial cells. This lipid is not usually secreted. Towards the end of gestation, characteristic changes in lipid metabolism take place (Mather, 1987), and abundant lipid synthesis and secretion commences after parturition. Thus, fat secretion is characteristic of lactation. A unique feature of cells cultured on EHS matrix is the secretion of milk fat globules and their accumulation within the lumina of differentiated cultures. This has not previously been documented in cultured mammary epithelial cells and is another indication that cells cultured on EHS matrix differentiate to a lactating phenotype. Milk fat globules are secreted from mammary epithelial cells by extrusion within an envelope of apical plasma membrane (Wellings *et al.* 1960; Bargmann, 1962; Wellings, 1969; Keenan and Dylewski, 1985; Mather, 1987), and this is also observed in cells cultured on EHS matrix. As has been described in lactating gland *in vivo*, milk fat globule formation appears to occur when intracytoplasmic lipid droplets approach the apical plasma membrane and become closely apposed to it. Evidence *in vivo* has suggested that cytoplasmic membranes may also contribute to the milk fat globule membrane (Wooding, 1977), and our observation of casein micelle-containing secretory vesicles surrounding cytoplasmic lipid droplets is consistent with this model. Previous studies have indicated that secretion of two milk fat globule membrane markers, butyrophilin and xanthine oxidase, is increased within cells seeded onto floating collagen gels (Lee *et al.* 1984) and in the lumina of cultures on EHS matrix (Barcellos-Hoff *et al.* 1989). Taken together, these results suggest that this experimental model should be useful for studying the unusual secretory mechanism(s) underlying milk fat globule formation.

Milk protein gene expression is correlated with cytodifferentiation in culture

We have previously reported that cultures of primary mouse mammary epithelial cells plated on a reconstituted basement membrane contain high levels of mRNA for the milk protein genes, β-casein (Li *et al.* 1987), transferrin (Chen and Bissell, 1987) and whey acidic protein (Chen

and Bissell, 1989), and that these cultures synthesize and secrete significant amounts of these proteins (Barcellos-Hoff *et al.* 1989; Chen and Bissell, 1989). Induction of tissue-specific gene expression in cells cultured on this extracellular matrix occurs in parallel with their reorganization into sealed spheres that resemble secretory alveoli in the mammary gland (Barcellos-Hoff *et al.* 1989). Although it was apparent from these earlier studies that the cells in alveolar structures in culture were typical of a secretory epithelium (apical/basal polarity, tight junctions, well-developed RER and Golgi apparatus), these characteristics are also seen in the pregnant gland; thus, a more detailed investigation of this cytodifferentiation has been necessary. In fact, we find that these highly differentiated epithelial cells are more similar morphologically to those of lactating mammary gland than they are to those of the mid-pregnant glands from which they were isolated. Most interestingly, *in situ* hybridization studies have revealed that it is precisely the differentiated secretory cells lining the lumina within the alveolar structures in our cultures that contain abundant mRNA for the specific milk protein, β-casein. The striking reorganization and functional segregation of secretory cells induced by the extracellular matrix provides further evidence that histiotypic architecture and concomitant cytoplasmic differentiation are important determinants of cell function. Thus, these studies confirm at the level of individual cells our hypothesis that cell structure is an important determinant of tissue-specific gene expression (Bissell *et al.* 1982; Aggeler *et al.* 1984; Bissell and Hall, 1987; Aggeler *et al.* 1988).

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