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## An appraisal of the utility of primary cell culture from sheep udders to investigate the control of mammary function

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### ABSTRACT

Clumps of mammary secretory epithelial cells were isolated from a lactating sheep udder and their mammary function in vitro was evaluated. Initially, the cells synthesized and secreted significant amounts of casein,  $\beta$ -lactoglobulin and at least 56 other minor proteins. However, the synthesis of these and almost all other cellular proteins had decreased dramatically by 24 h in culture. This was accompanied by a decrease (23% of initial levels) in RNA recovered from the cells and a decrease (4% of initial levels) in the relative abundance of  $\alpha$ -lactalbumin RNA. Secretion of what appears to be a casein species was increased in the presence of a mixture of foetal calf serum, prolactin, insulin, and hydrocortisone up to 24 h in culture. These agents also stimulated cellular growth and proliferation over 8 days. However, no hormonal effect was observed on milk protein synthesis or gene expression. Thus, this mammary cell culture system has only a limited use as an in vitro model of mammary function.

**Keywords:** mammary, lactation, protein-synthesis, secretion.

### INTRODUCTION

It would be of considerable benefit to be able to manipulate milk production in dairy cows so as to enhance its value. This could, for instance, result in milk with reduced water content, reduced fat content, or increased concentrations of desirable variants of milk proteins (Wilkins, 1991; Hennighausen, 1992). These characteristics could be produced by genetic or biochemical alteration of the cow's mammary biology, however, these approaches require a detailed understanding of mammary function, particularly how the expression of milk protein genes, as well as milk protein synthesis and secretion, is controlled. These goals would be greatly facilitated by the use of an in vitro model of mammary function such as tissue culture, primary cell culture or mammary cell lines in which the control of mammary function is maintained and which can also be readily manipulated. Such an in vitro model could be used to detect changes in the synthesis of proteins that may accompany changes in mammary function. Similar models have been used extensively to study lactation in rodents, for example (Matusik and Rosen, 1978; Eisenstein and Rosen, 1988; Streuli *et al.*, 1991), but their use in ruminants has to date been somewhat limited. We have evaluated isolated clumps of mammary cells from lactating sheep for their ability to maintain synthesis and secretion of milk proteins, expression of milk protein genes and their hormonal responsiveness in vitro.

### RESULTS

#### Synthesis and secretion of milk proteins: Analysis by one-dimensional electrophoresis

Clumps of mammary epithelial cells were prepared from

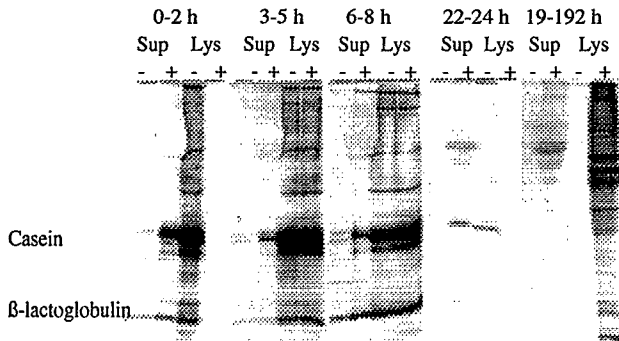
udder tissue from a lactating sheep as described elsewhere in this volume (Davis *et al.*, 1993) and incubated in the presence of a mixture of galactopoietic agents (5  $\mu$ g/ml insulin, 1  $\mu$ g/ml prolactin, 1  $\mu$ g/ml hydrocortisone and 1  $\mu$ g/ml prostaglandin E<sub>2</sub>) and 10% foetal calf serum. The cells were metabolically labelled with [<sup>35</sup>S]methionine for 2 h at varying times thereafter using a previously described procedure (Sadowski *et al.*, 1992). The labelled intracellular and secreted proteins were separated by electrophoresis and detected by autoradiography.

During the first few hours in culture, proteins were extensively labelled with <sup>35</sup>S, indicating that initially the cells were metabolically active. However, they had lost most of this activity by 24 h (Fig. 1). Despite this, proteins of molecular weight 29 and 18 kilodaltons (almost certainly casein and  $\beta$ -lactoglobulin) were secreted throughout this period, although in decreasing amounts. Furthermore, the secretion of what appears to be a casein species was markedly enhanced in the presence of the galactopoietic agents and foetal calf serum (Fig. 1). Thus, at least for one milk protein it appears that secretory activity is maintained over this time and remains hormonally responsive.

Initially, the cells were plated as large clumps. However, by 8 days in culture in the presence of the galactopoietic agents and foetal calf serum the cells had taken on a "cobblestoned" morphology interspersed with large dome-like areas, typical of cultured rodent mammary cells that are synthesizing and secreting milk proteins (Danielson *et al.*, 1984). Although many intracellular proteins were metabolically labelled at this time, no milk proteins were unequivocally detected either intracellularly or in the culture supernatant. This analysis does not rule out the presence of trace amounts of milk proteins. To address this, intracellular and secreted proteins from 8 day cultures were immunoblotted using antibodies raised against

sheep casein and  $\alpha$ -lactalbumin. No milk proteins were specifically detected (result not shown).

**FIGURE 1:** Protein synthesis in mammary cells isolated from a lactating sheep. Proteins were metabolically labelled for 2 h at the indicated times after initiating the incubation, either in the presence (+) or absence (-) of 10% foetal calf serum, 1  $\mu$ g/ml prolactin, 5  $\mu$ g/ml insulin, 1  $\mu$ g/ml hydrocortisone and 1  $\mu$ g/ml prostaglandin E<sub>2</sub>. Labelled proteins secreted into the culture medium (sup) and those remaining inside the cells (lys) were analysed by SDS electrophoresis followed by autoradiography. The two blank lanes (0-2 h and 22-24 h cellular lysate proteins labelled in the presence of hormones and foetal calf serum) are due to samples being lost during the experiment.



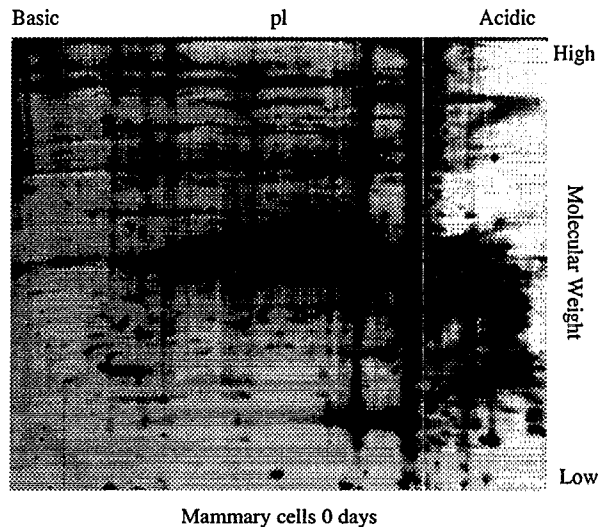
### Synthesis and secretion of milk proteins: Analysis by two-dimensional electrophoresis

The synthesis and secretion of metabolically labelled proteins in isolated mammary cells was examined in greater detail using two-dimensional electrophoresis on large-format gels using a previously described procedure (Young *et al.*, 1983). Analysis of proteins secreted during the first 2 h of incubation reveals at least 56 major milk proteins, variants thereof or other secreted minor proteins. These proteins are indicated in Fig. 2b. The secretion of at least 32 of these (arrowed in Fig. 2b) remains unaltered in the presence of galactopoietic agents and foetal calf serum. However, secretion of 21 other proteins (circled in Fig. 2b) is significantly altered in the presence of foetal calf serum alone. Furthermore, what appear to be 3 positively charged alternate isoforms of  $\beta$ -lactoglobulin are secreted only in the presence of a mixture of the galactopoietic agents and foetal calf serum (proteins within triangles in Fig. 2b). These results indicate that rather than a general stimulation of secretion, components of the hormone mixture have specific effects on the secretion of particular proteins.

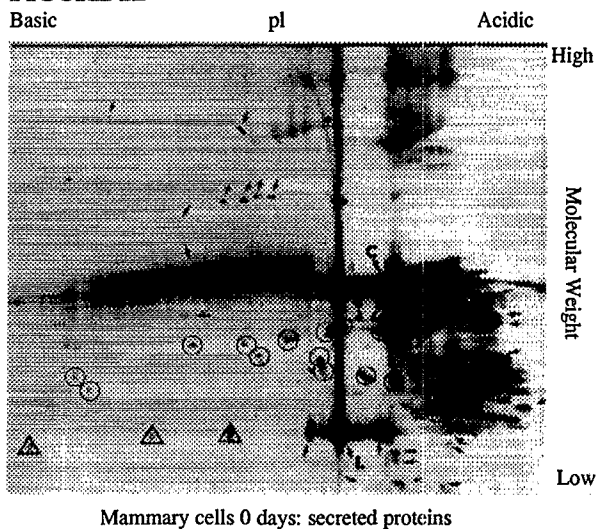
These experiments also revealed a striking change in the pattern of intracellular proteins after 8 days in culture. Initially, casein and  $\beta$ -lactoglobulin isoforms were the predominant intracellular and secreted proteins (Fig. 2A and B), however, by 8 days these proteins are no longer synthesized and a protein with a similar molecular weight, pI and abundance in fibroblasts to actin is the predominant intracellular protein (the most abundant protein in Fig. 2C). Furthermore, the overall pattern of protein synthesis is similar to a mouse mammary derived fibroblastic cell line, C127 (Fig 2D). Thus, it seems likely that after several days in culture mammary fibroblasts overgrow the secretory cells.

**FIGURE 2:** Two-dimensional electrophoretic analysis of metabolically labelled proteins. Mammary cell proteins were labelled from 0-2 h in culture and recovered (a) from the cells, and (b) from the culture supernatant. (c) Mammary cells were also cultured for 8 days in the presence of the galactopoietic agents, then labelled for 2 h and the proteins within the cells analysed. (d) cellular proteins from C127 mammary fibroblasts labelled for 2h. The casein (C) and  $\beta$ -lactoglobulin (L) spots are indicated on Fig. 2b. Arrowed proteins in Fig. 2b are secreted to about the same extent in the presence or absence of the hormones and foetal calf serum. The rate of secretion of the circled proteins were markedly altered in the presence of foetal calf serum alone. Secretion of the 3 proteins within triangles occurred in the presence of a mixture of the hormones and foetal calf serum, but not foetal calf serum alone.

**FIGURE 2A**



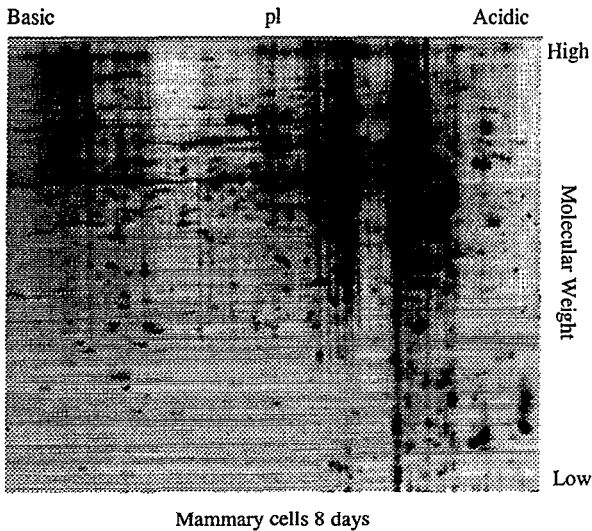
**FIGURE 2B**



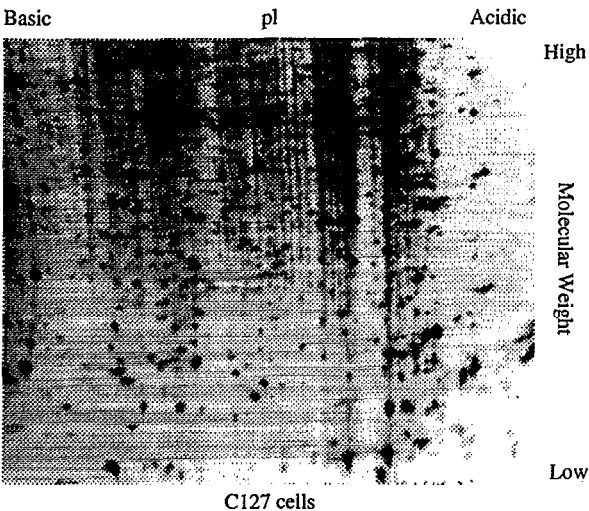
### RNA levels in isolated mammary cells

To determine whether the decrease in synthesis of proteins during the first day in culture is a consequence of decreased RNA levels, RNA was isolated from the cells during this time. Total RNA recovered decreased to about 23% of initial levels by 22 h and the presence of the galactopoietic agents and foetal calf serum, or the presence of an extracellular matrix-derived gel (matrigel, Integrated Sciences) had no significant effect on this (Fig. 3A). When equal quantities of RNA were analyzed by Northern blotting, the

**FIGURE 2C**



**FIGURE 2D**

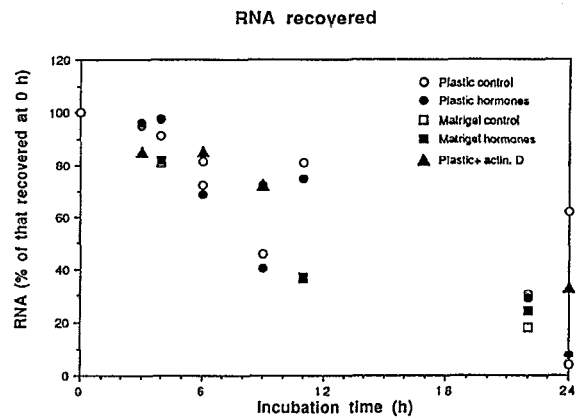


relative abundance of  $\alpha$ -lactalbumin RNA decreased to 4% of initial levels by 22 h (Fig. 3b). Although this could indicate a faster shut-down in mammary function compared with the cell's general metabolic activity, the results are also consistent with a complete shut-down of transcription during isolation of the cells if  $\alpha$ -lactalbumin RNA is less stable compared with ribosomal RNA. In any case, transcription to RNA appears to decrease soon after cell isolation independent of the presence of galactopoietic agents.

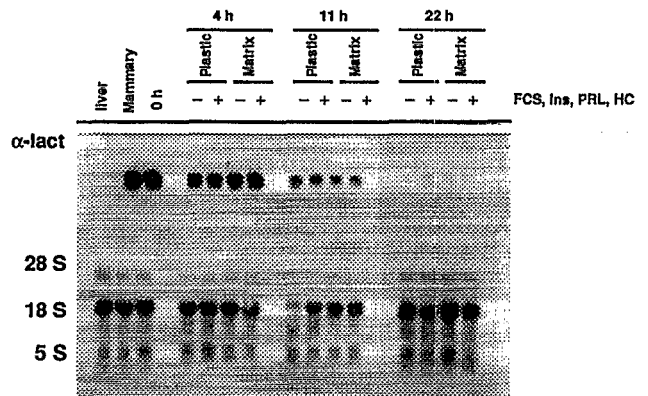
If the cells do actively transcribe RNA in culture, RNA abundance should be further decreased after exposing the cells to an inhibitor of transcription such as actinomycin D. A recent such experiment showed no effect by actinomycin D on  $\alpha$ -lactalbumin RNA abundance (result not shown). Thus, it seems that active transcription of at least this milk protein gene is lost in the process of cell isolation and is not regained in culture. Consequently, the decline in synthesis of mammary proteins in culture may well be due to translation from a declining pool of pre-existing RNA.

**FIGURE 3:** RNA abundance in isolated mammary cells. Cells were plated onto 3 cm diameter wells of a cell culture plate which had previously been coated with matrigel (Integrated Sciences), an extracellular matrix-derived gel (lanes and datapoints labelled "matrix") or were plated directly onto plastic (lanes and datapoints labelled "plastic"). (a) RNA was recovered from the cells at the indicated incubation times in the presence (hormones) or absence (control) of foetal calf serum, insulin, prolactin and hydrocortisone, or in the presence of actinomycin D (actin. D) using the method of Gough (Gough, 1988). In this procedure cells were lysed in NP-40, nuclei pelleted and the cytosolic RNA precipitated from the supernatant (in an SDS/urea solution) after phenol:chloroform extraction. Some RNA samples were recovered by an alternate procedure in which the cells were lysed in guanidinium isothiocyanate and RNA precipitated from the acidified solution after phenol:chloroform extraction (Chirgwin *et al.*, 1979). The data were collated from several different experiments. (b) Northern blot analysis of  $\alpha$ -lactalbumin and ribosomal RNA. Equal (10  $\mu$ g) quantities of RNA from cells cultured in the presence (+) or absence (-) of the mixture of hormones and foetal calf serum was loaded onto an agarose/formaldehyde gel, electrophoresed and transferred to nitrocellulose. For comparison, 10  $\mu$ g RNA from ovine liver and mammary tissue were also analysed.  $\alpha$ -lactalbumin and ribosomal RNA were detected by hybridisation with radiolabelled DNA probes.

**FIGURE 3A**



**FIGURE 3B**



**DISCUSSION**

Taken together, these results show that primary mammary cells from lactating sheep rapidly lose their ability to synthesize milk proteins when they are incubated in culture, independent of the presence of galactopoietic agents, and for at least  $\alpha$ -lactalbumin this occurs as a consequence of declining RNA levels, probably due to a shut-down of transcription during cell isolation. One possible explanation for this is that the mammary epithelial cells were perturbed, damaged or

made hormonally unresponsive during their isolation. Thus, the loss of milk protein synthesis may either be the consequence of a traumatic perturbation of cellular metabolism, the loss of hormonal stimuli or the loss of some other facilitative interaction with the extracellular environment. Another possibility is that during isolation the cells have been triggered to undergo a programmed shut-down of function, similar to that which occurs *in vivo* during mammary involution. However, the normal involution process involves a dramatic increase in expression of lactoferrin and its secretion. An increase in expression, synthesis or secretion of lactoferrin was not observed by metabolic labelling or on Northern blots. Further work would be required to clarify which of these possible explanations most accurately describes the mechanism by which mammary function is lost.

In conclusion, milk proteins are synthesized and secreted in relatively large quantities in isolated clumps of mammary cells, but this mammary function was lost and could not be regained after 24 h in culture. Interestingly, the secretion of what appears to be a casein species is greater in the presence of galactopoietic agents and foetal calf serum. However, neither synthesis of mammary proteins nor RNA abundance are influenced by the presence of the hormones and foetal calf serum. Taken together, these results suggest that this mammary cell culture system has only a limited use as an *in vitro* model of mammary function.

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