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journal or publication title	Journal of Integrated Field Science
volume	3
page range	67-72
year	2006-03
URL	http://hdl.handle.net/10097/30961

The Long Road to a Representative *In Vitro* Model of Bovine Lactation

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Introduction

The biology of the mammary gland of the dairy cow has long been the focus of scientific investigation. The mammary gland represents a unique system for the study of organogenesis, and it has the distinctive ability to enter a cyclical process of development and regression along with successive cycles of pregnancy and lactation. As yet, however, the mechanisms controlling this are poorly understood, particularly for cattle (Chotteau-Lelievre *et al.* 2003). Moreover, during lactation the mammary gland is involved in the mass transfer of nutrients from the blood into milk, though the transport of a number of these is poorly understood. For example, the calcium in cows' milk is important in human nutrition, but relatively little is known about its transfer into milk (Neville, 2005). Furthermore, *in vivo* experimentation has not been able to shed a great deal of light on these mechanisms (e.g. Barlet *et al.* 1992).

There are two fundamental disadvantages of studying lactation *in vivo*: Firstly, because of the ability of mammals to maintain a constant internal environment, treatments that are applied can have systemic effects that confound the net effect at the mammary gland. Controlling the environment of the milk secreting epithelial cells *in vivo* in a predictable way is therefore very difficult. In contrast, an accurate model of mammary function would allow the mammary component to be isolated and its environment to be controlled independently of systemic effects. Secondly, there is an unease amongst the general public (in the UK at least), over the use of animals for experimentation, particularly when that research is not for medical purposes (e.g. MORI, 2002). Taking these arguments together, lactational physiologists would therefore find a representative model of bovine lactation of major use in gaining a greater understanding of mammary biology.

A Brief Critique of In vitro *Systems of Ruminant Mammary Gland Biology*

A number of attempts have been made to replicate the function of the bovine mammary gland *in vitro*. The methodologies involved have been reviewed (Blum *et al.* 1989; Ip and Darcy, 1996; Matitashvili *et al.*, 1997; Shaw *et al.* 2004). The earliest attempts to model the mammary gland in culture used rodent explants containing whole alveoli (Elias, 1957). This methodology has been used several times in the intervening years for cattle tissue (e.g. Feuermann *et al.* 2004; Yang *et al.* 2005). The major advantage of explant culture is that the cellular composition of the mammary tissue, including the extracellular matrix, is (at least initially) similar to that of *in vivo* tissue. Accordingly, the effects of hormones and growth factors etc. may be investigated under a comparatively native environment. However, the interpretation of the results of explant cultures can be difficult. Firstly, there is the potential of carry-over effects from the animal from which the mammary tissue was obtained, including latent growth factors and hormones activated during the incubation. Secondly, there is a difficulty of identifying the primary cellular target of factors added to the media. An additional difficulty is that of determining changes that occur in each cell type within the tissue. Finally, explants remain viable in culture only for a limited period of time.

An alternative approach is to use mammary epithelial cells (MEC) that have been isolated and separated from the extracellular matrix of mammary tissue. These are then plated onto cell culture-ware, usually existing as a monolayer of cells. Freshly isolated cultures of primary MEC have been used extensively in this way. More recently, immortalised as well as clonal lines of bovine MEC have also been used. Methodologies for the maintenance of primary cultures of bovine MEC were first developed over two decades ago (Mackenzie *et al.* 1982; 1985), and since then there has been a constant development of techniques to replicate the biology of the mammary

gland *in vitro*. However, even now, a good many of these attempts may be regarded as deficient in one or more of the following ways:

Experiments are often performed on cells that are relatively undifferentiated (that is, they do not have the intracellular biochemical processes that occur in the natural state). This is because many studies have used MEC plated on cell culture plastic-ware (e.g. Cheli *et al.* 2003). When MEC are cultured in this way, they form a monolayer attached to the plastic that excludes the possibility of cellular polarisation. Crucially, the cells in this state generally do not synthesise any milk components, nor do they have the cellular responses of MEC found *in vivo* (Blum *et al.* 1989).

Another problem of attempts to replicate the biology of the mammary gland *in vitro* is that the cells used have often been transformed (that is, they are unnaturally immortal; e.g. Silva *et al.* 2002). Immortalisation can occur spontaneously, or it may occur as a result of deliberate transfection of viral genes. Either way, immortal cell lines have a reduced dependence on hormones and factors for growth in culture, due in part to their abnormal secretion of these or other growth factors (Todaro and Delarco, 1978). Furthermore, immortalised cell lines often have other abnormalities not seen in the untransformed cell (Matitashvili *et al.* 1997). For example, the immortal MAC-T bovine mammary cell line, most often used in bovine mammary research *in vitro*, is not a single homogeneous cell type (Zavizion *et al.* 1994). Additionally, this cell line has very low levels of milk specific protein production, relative to the levels seen in untransformed cultures (German and Barash, 2002). There is also some evidence that the MAC-T cell line is not dependent on the *in vivo* factors (hormones or extracellular matrix) known to regulate differentiation *in vivo* (Huynh *et al.* 1991; Berry *et al.* 2003), suggesting that their receptor mechanisms differ from the *in vivo* state. Studies of other immortal bovine MEC lines, 'BME-UV' and 'HH2a', have also indicated that these too have an abnormal physiology when attempts are made to bring them to the differentiated state (Matitashvili *et al.* 1997)

To date, attempts to model the biology of the bovine mammary gland have nearly always used MEC that have been cultured in isolation from the other cells types known to be important to its development and function. For example, the fibroblast growth factors (secreted by fibroblasts) are thought to greatly influence the proliferation and morphogenesis of MEC *in vivo* (Powers *et al.* 2000). Additionally, fibroblasts are thought to secrete at least part of the extracellular matrix and basement membrane for the parenchyma, and are a site of action of various hormones, in addition to synthesising their own growth factors (Hovey *et al.* 1999). During the early stages of development of the mammary gland, adipose tissue is thought to be crucial. However, despite this, these cell types are very rarely included in *in vitro* culture systems.

A further problem relates to the fact that the hormones and growth factors that have been shown to regulate MEC function *in vivo* are often absent from the culture media (or are undefined in the media because of the use of foetal bovine serum (FBS)). FBS is often used because it contains (undefined) cell attachment factors, growth factors and nutrients. The removal of FBS from culture media has the advantage of allowing the media to be free of (unknown) confounding growth factors, hormones and other components. Additionally, FBS can be of variable composition between batches, and it can be a way of introducing infection into cell cultures.

Some *in vitro* investigations have used pre-formed extracellular matrices (e.g. that from the Engelbreth-Holm-Swarm mouse sarcoma). These result in substantial morphological differentiation of the MEC as well as milk component synthesis (e.g. Rose *et al.* 2002). These matrices are rich in the extracellular proteins, which enable MEC to form lobule like structures, reminiscent to those seen *in vivo*, into which milk components are secreted (Rose *et al.* 2002; McConochie 2004a). However, the problem is that the contents of the lumina are difficult to obtain without destroying the lobule. A further problem is that as milk components accumulate in the lumina, there may be feedback inhibition of further milk synthesis (Peaker and Wilde 1996). Finally, Matrigel is also rich in a range of murine growth factors that may confound experimental results obtained.

Cell Culture Insert Methodology

Virtually all reports of *in vitro* models of bovine mammary function published to date can be criticised in one or more of the ways outlined above. In Aberystwyth, we have pursued an alternative

methodology that avoids some of these criticisms. We have isolated an untransformed (mortal) clonal cell line from lactating mammary tissue and have frozen it in numerous aliquots (Rose *et al.* 2002). These aliquots of cells can be defrosted as required and used for a number of passages. This methodology allows for several years of experimentation on the same batch of cells. Nevertheless, the cells are not immortalised by transformation with viral genes.

The cells are plated onto two-dimensional, porous, cell culture well-inserts. These allow free and repeated access to products secreted by the cells, whether to their apical or basolateral side (Delabarre *et al.* 1997; McConochie *et al.* 2004b, 2005). Additionally, because the cells are plated onto a porous membrane, the cells may be treated with different media on each side, reflecting more accurately the *in vivo* situation. Moreover, it is possible to co-culture other cell types, such as fibroblasts, in the lower chamber while the MEC are on the porous membrane.

We plated clonal bovine MEC onto collagen I coated porous membrane inserts and treated them with media in both the upper and lower chambers containing Dulbecco's Modified Eagle's Medium supplemented with either 10 ml/l of FBS or 10ml/l FBS with 5 mg/l each of the lactogenic hormones prolactin, dexamethasone and insulin (PDI). We found that significantly greater levels of the milk proteins α-casein and α-lactalbumin were produced with the PDI treatment (Figure 1). Furthermore, a substantially greater proportion of the α-lactalbumin secreted was found in the upper chamber (Figure 1b), suggesting that a degree of polarisation had occurred. However, in contrast, the α-casein was found to a significantly greater extent in the lower chamber (Figure 1a). Despite this, in terms of the concentration of α-casein in the media, approximately the same concentration of α-casein was found in the lower chamber as in the upper chamber. The greater total amount in the lower chamber therefore reflects the greater volume of media present there. Accordingly, we also concluded that α-casein might have had a greater mobility between the chambers than the α-lactalbumin. Certainly, the cells on the porous membrane were not wholly confluent, but did recede from the edges slightly in this experiment, allowing diffusion from the upper chamber to the lower chamber. The reason for the relative immobility therefore of the α-lactalbumin is unclear, but it is known that α-lactalbumin is at least partially associated with the apical membrane of MEC (Sasaki *et al.* 1978). Finally, it was noticeable in this experiment that even without the lactogenic hormones (but with the inclusion of the FBS), a degree of milk specific protein secretion was observed, possibly suggesting that FBS has some lactogenic properties.

Next we determined the effect of the coating of the porous membrane (either no coating, collagen I coating, or laminin coating) on the secretion of α-casein by the MEC clone when cultured with either FBS alone, the lactogenic hormones (PDI) or FBS plus PDI. The surprising finding was that there was

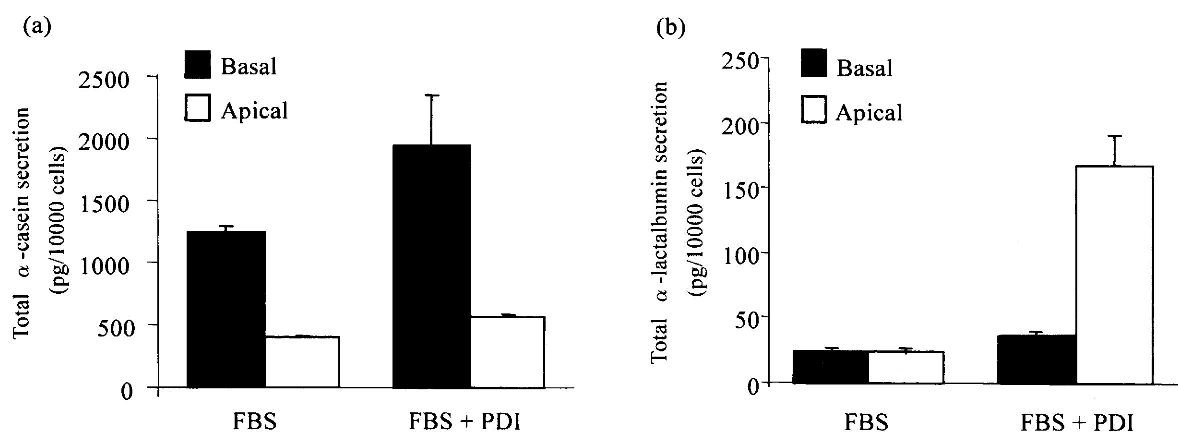

Fig. 1. Secretion of (a) α-casein and (b) α-lactalbumin into the apical (□) and basal (■) chambers by clonal bovine MEC plated onto collagen-I coated porous membrane inserts in media containing either 10 ml/l of foetal calf serum (FBS) or 10 ml/l of FBS supplemented with 5 mg/l of prolactin, dexamethasone and insulin.

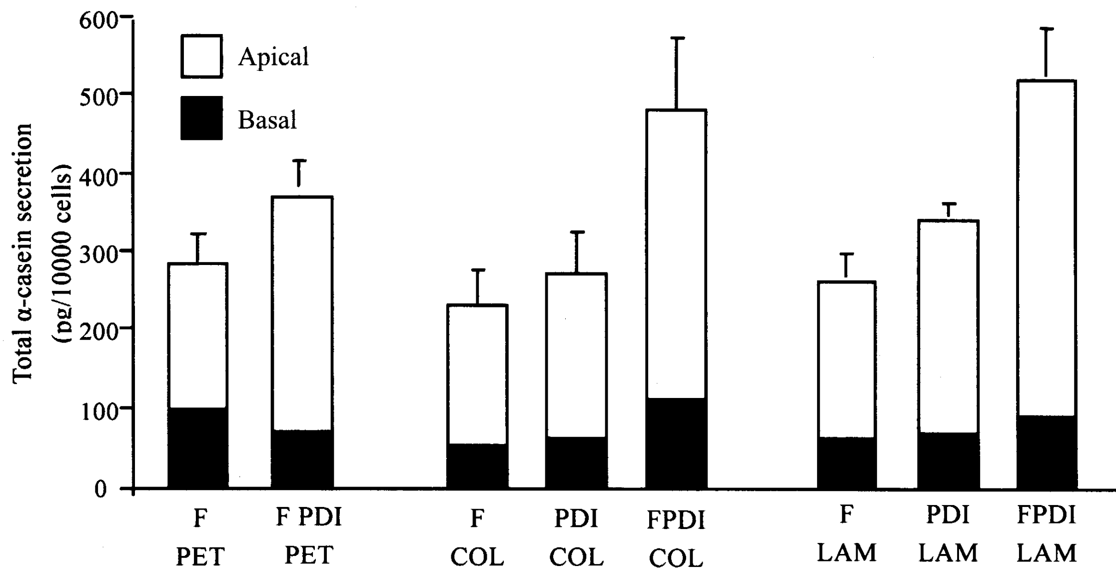

Fig. 2. Secretion of α-casein into the apical (□) and basal (■) chambers by clonal bovine MEC plated onto either uncoated (PET), collagen-I coated- (COL), or laminin coated- (LAM) porous membrane inserts in media containing either 10 ml/l of foetal calf serum (F), 5 mg/l of prolactin, dexamethasone and insulin (PDI), or both (FPDI).

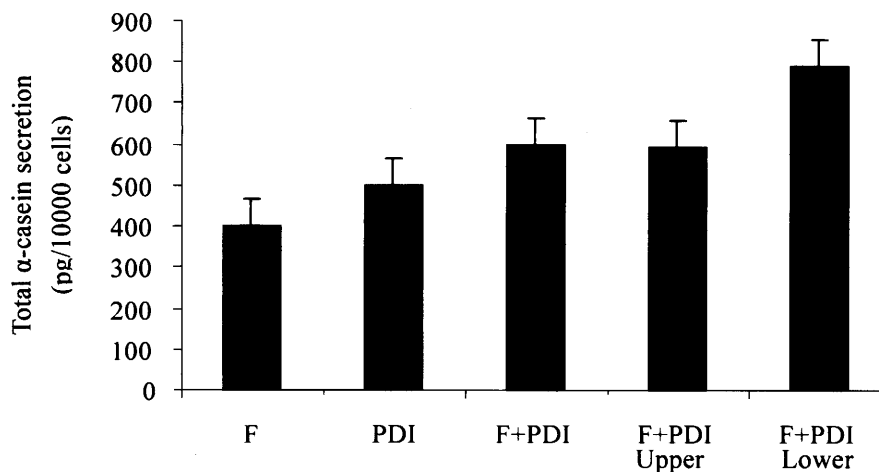

Fig. 3. Total secretion of α-casein by clonal bovine MEC plated onto collagen-I coated- porous membrane inserts. The media contained either 10 ml/l of foetal calf serum in both the upper and lower chamber (F), 5 mg/l of prolactin, dexamethasone and insulin in both the upper and lower chamber (PDI), F and PDI in both the upper and lower chamber (FPDI), F and PDI in the upper chamber only (FPDI upper), or F and PDI in the lower chamber only (FPDI lower).

no effect of the coating of the porous membrane on the secretion of α-casein (Figure 2). All treatments resulted in the secretion of α-casein to a similar extent. Again, milk protein was secreted in the absence of lactogenic hormones, but in the presence of FBS. However, the lactogenic hormones alone resulted in generally (but non-significantly) higher levels of secretion of α-casein. The combination of FBS and the lactogenic hormones resulted in significantly higher rates of secretion of α-casein, relative to either treatment alone. However, we found that this occurred to a lesser extent for the uncoated inserts. When we stained the uncoated membranes for the presence of laminin and collagen I, we found that both proteins had been deposited on the membrane. As these proteins were absent from the

uncoated membranes we concluded that these extracellular matrix proteins had been secreted by the MEC. *In vivo* it is thought that fibroblasts are largely responsible for the secreting extracellular matrix.

In a further experiment we again measured the total α-casein secretion by the MEC plated onto collagen-I coated- porous membrane inserts. In this experiment, the media contained either 10 ml/l of foetal calf serum, 5 mg/l of prolactin, dexamethasone and insulin (PDI) or F plus PDI in both the upper and lower chambers of the cell culture well inserts. A further two treatments had the F plus PDI in the upper chamber only, or F plus PDI in the lower chamber only, with unsupplemented DMEM in the other chamber, respectively. In this experiment, the secretion of α-casein was significantly greater when F+PDI was in the lower chamber only, relative to being in the upper chamber only, or in both chambers (Figure 3). This may indicate an increased level of polarisation when hormones and growth factors are present on one side only.

Conclusions and Perspective

While the methodology we discuss in the second half of this review is clearly open to further improvement, in particular with regard to the continued need by the cells for FBS, it nevertheless does represent an improvement over some of the methodology discussed in the earlier part. In particular, we have shown that the cells are responsive to lactogenic hormones and that the cells do reach a state of functional differentiation when plated on the porous membrane. Furthermore, the insert methodology allows the repeated sampling of the substances synthesised by the cells without the destruction of the morphological integrity of the cells. The methodology allows different treatments to be applied to the cells in the upper and lower chambers, and it also allows another cell type to be cultured in the lower chamber, separate from but in reasonably close proximity to the MEC. Unfortunately, our cells retain the requirement for FBS. This is required for the attachment of the cells to the porous membranes and thereafter for elevated levels of milk protein synthesis. Further research will concentrate on eliminating FBS from the culture media.

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