The lactation cycle of the fur seal

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Introduction

The fur seal is a mammal with an unusual ability to turn its milk production on and off without significantly altering the gross morphology of the mammary gland. This atypical lactation cycle is due to the fact that maternal foraging and infant nursing are spatially and temporally separate (Bonner, 1984). Maternal care involves the suckling of offspring over a period of at least 4 months, but lactation can extend to more than 12 months. Following a perinatal fast of approximately 1 week, females depart the breeding colony to forage at sea and, for the remainder of lactation, alternate between short periods ashore suckling their young with longer periods of up to 4 weeks foraging at sea. Whilst foraging at sea, milk production in the fur seal mammary gland either ceases or is reduced (Arnould & Boyd, 1995b).

Gross composition of seal milk differs from that of other mammals (Davis et al. 1995). For example, seal milk is richer in lipid than that of terrestrial mammals (Jenness & Sloan, 1970; Bonner, 1984; Oftedal, 1984; Baker, 1990; Arnould & Hindell, 1999). This feature allows the rapid transfer of energy to the sucking young in order to build up an insulating blubber layer, which also serves as an energy store once the mother begins to leave for foraging at sea (Trillmich & Lechner, 1986). The lipid content of fur seal milk is related to the duration of the proceeding foraging trip (Arnould & Boyd, 1995b) suggesting that the fat content of fur seal milk is adapted to satisfy the pups' need for sustaining normal activity during maternal absence (Arnould & Trillmich, 1985). This pattern of lactation means mothers have long periods with no sucking stimulus and can transfer high energy milk rapidly while on land so as to minimize time away from foraging grounds. This type of lactation is unique amongst mammals owing to the extreme duration of inter-suckling bouts and the rapid rate of transfer while suckling.

The composition of fur seal milk

Lipid

Gross composition of fur seal milk differs from that of other mammals (Davis et al. 1995). For example, fur seal milk

has up to 50% lipid which is considerably more than that of terrestrial mammals (Jenness & Sloan, 1970; Bonner, 1984; Oftedal, 1984; Baker, 1990; Arnould & Hindell, 1999). This feature allows the rapid transfer of energy to the sucking young in order to build up an insulating blubber layer which also serves as an energy store once the mother begins to leave for foraging at sea (Trillmich & Lechner, 1986). The lipid content of fur seal milk is related to the duration of the proceeding foraging trip (Trillmich & Lechner, 1986), suggesting that the fat content of fur seal milk is adapted to satisfy the pups' need for sustaining normal activity during maternal absence (Arnold & Trillmich, 1985). The fatty acid profiles of milk lipid have been determined for a number of seal species, revealing changes associated with either diet or stage of fasting (Van Horn & Baker, 1971; Trillmich et al. 1988; Ochoa-acuna et al. 1999; Georges et al. 2001).

Protein

The protein concentration of fur seal milk ranges from 10 to 18% and is among the highest of any mammal (Trillmich & Lechner, 1986; Arnould & Boyd, 1995b; Davis et al. 1995; Arnould & Hindell, 1999; Goldsworthy & Crowley, 1999; Georges et al. 2001). The proportions of casein to whey protein vary considerably between species (Anderson et al. 1985), including the pinnipeds (Ashworth et al. 1966; Trillmich et al. 1988). A recent study (Cane et al. 2005) shows that the average casein to whey ratios for the Australian and Antarctic fur seals (1·2 and 0·69, respectively) are similar to the ratio reported for the Northern fur seal, *Callorhinus ursinus* (Ashworth et al. 1966), but lower than the ratio of 2·1 reported for the Galapagos fur seal, *A. galapagoensis* (Trillmich et al. 1988).

Few milk proteins have been identified in pinnipeds (Ashworth et al. 1966; Trillmich et al. 1988; Ronayne de Ferrer et al. 1996). Our recent studies (Cane et al. 2005) showed the casein micelle of the Australian fur seal is composed of five caseins whereas Ronayne de Ferrer et al. (1996) observed four casein bands after SDS-PAGE of milk proteins from the Southern elephant seal. Their study also described five distinct whey proteins, but only serum albumin was identified according to its mobility in the gel (Ronayne de Ferrer et al. 1996). Cane et al. (2005) reported

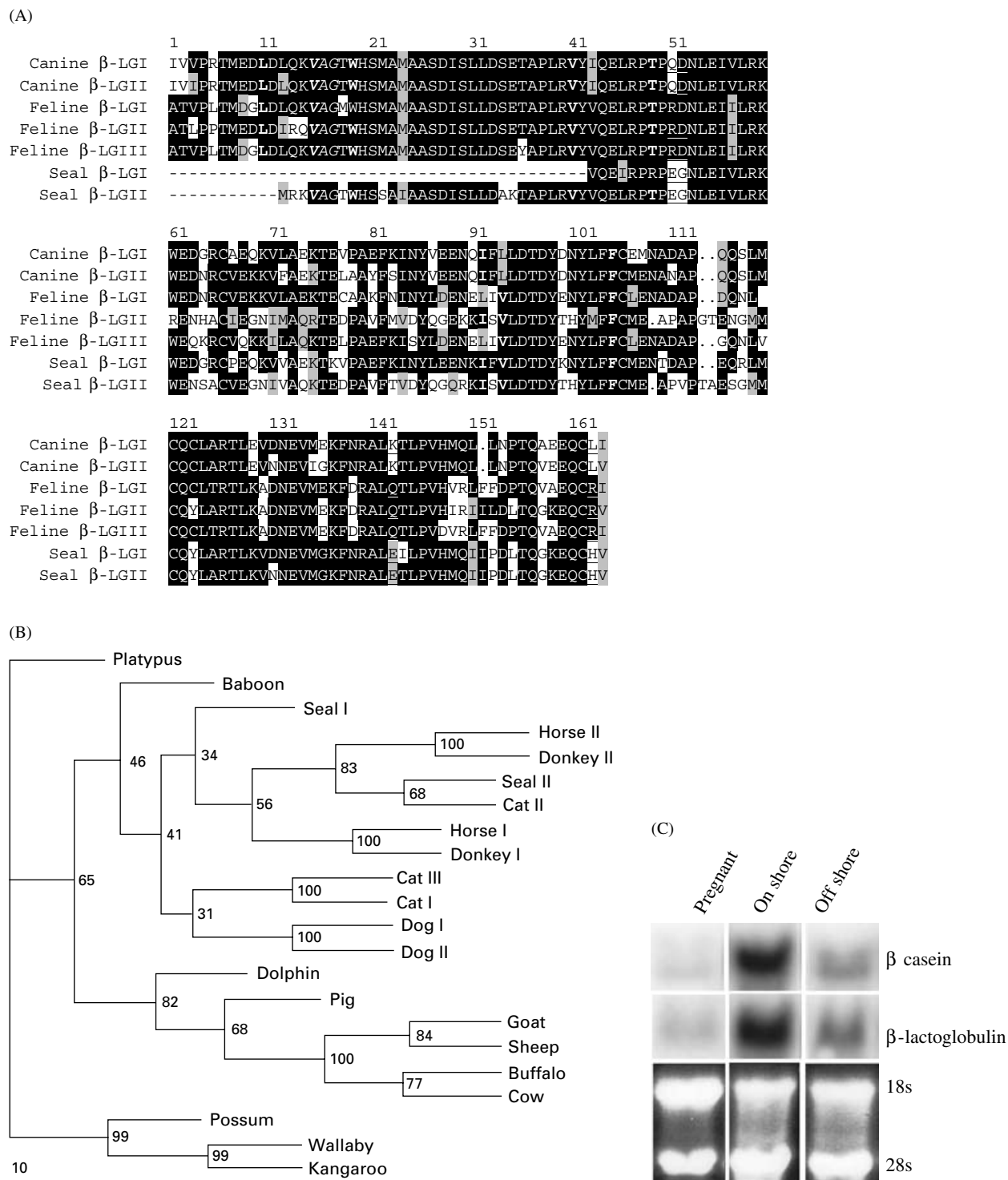*For correspondence; e-mail: jasharp@unimelb.edu.au

(A)

(B)

(C)

Fig. 1. (A) Comparison of primary peptide sequences of fur seal, canine and feline β-lactoglobulins (β-lg). Fur seal β-lg nucleotide sequences were translated and aligned with the sequences of canine β-lg I and II and feline β-lg I, II and III. Residues shaded in black are identical between sequences aligned, while grey shading indicates conservative substitutions. Residues conserved in the sequences of each species, but are different between the three groups are underlined. The italicized *VAG* at position 15 is characteristic of all β-lg except the kangaroo β-lg-like protein. Residues printed in bold make up the proposed binding site of retinol (amino acids are not conserved in all β-lg, but substitutions maintain the hydrophobic nature of the binding site, e.g., Val^{92}Ile seen in feline sequences also). Gaps (.) inserted to assist with alignment. (B) Phylogenetic relationship of the β-lg proteins, generated from alignment of 121 amino acid region common to all species (aa 43–163 shown in (A)). Length of the lines from the nodes indicate evolutionary distances. Bootstrap values are shown. (Acc. nos. Horse P02758, P07380; Cow P02754; Sheep P67976; Pig P044119;

that ten distinct whey bands from the milk of the Australian fur seal could be distinguished following electrophoretic separation, and N-terminal sequencing showed the majority of the protein to be β-lactoglobulin (β-lg), similar to that reported for ruminant, canine and dolphin milks (Bell et al. 1981; Hambling et al. 1992; Kushibiki et al. 2001). At least three isoforms of the protein appear to be secreted in the milk.

Although the biological function of β-lg remains to be determined, its presence in the milk of several mammals has suggested a nutritional role, supported also by its ability to interact with a great variety of hydrophobic ligands, such as retinol (Futterman & Heller, 1972; Dufour et al. 1990; Dufour & Haertle, 1991; Cho et al. 1994; Narayan & Berliner, 1997; Lange et al. 1998), fatty acids and triglycerides (Dufour et al. 1990; Frapin et al. 1993; Narayan & Berliner, 1997; Wang et al. 1997; Qin et al. 1998; Wu et al. 1999; Zsila et al. 2002) leading to speculation that β-lg may enhance vitamin A uptake in sucking offspring (Said et al. 1989; Kushibiki et al. 2001). The β-lg-retinol complex is known to bind receptors in the intestine of suckling calves (Perez et al. 1989) and may be involved in lipid metabolism, possibly by enhancing lipase activity (Perez et al. 1992). This proposed function of β-lg would be of significance with regard to fur seal metabolism, given that milk lipid accounts for up to 50% or more of the total composition of fur seal milk (Oftedal, 1993; Arnould & Hindell, 1999).

Carbohydrate

While carbohydrates are found in the milk of most phocids (true seals) (Messer et al. 1988) only trace amounts are present in the milk of otarrids (fur seals) (Dosako et al. 1983; Urashima et al. 2001). Lactose, the major carbohydrate in the milk of most mammalian species (Oftedal, 1993) is either absent or present in extremely low concentrations in the milks of fur seals (Schmidt et al. 1971; Dosako et al. 1983; Messer et al. 1988; Urashima et al. 2001). It has previously been suggested that lactose and oligosaccharides are not found in the milk of the Australian fur seal (Urashima et al. 2001). However, milk of this species contains inositol, a sugar known to promote infant growth (Arnould & Boyd, 1995b). In other mammals, milk that is low in lactose has relatively higher concentrations of sodium and potassium (along with chloride), in order to maintain osmolality (Oftedal, 1993). The sodium and potassium concentrations in the milk of fur seal species are comparatively higher (Cane et al. 2005) than the concentrations in the milk of terrestrial species such as humans and cows (Green et al. 1980; Schryver et al. 1986; Nicholas & Hartmann, 1991).

It is interesting that while the milk of the Tammar wallaby (*Macropus eugenii*) contains less than 1% (w/v) carbohydrate in late lactation, and the carbohydrate moiety is exclusively monosaccharides (Green et al. 1980). It is also at this late stage of lactation that milk volume increases and significant changes in sodium and potassium concentrations occur, whereby an increase in their concentrations is evident as carbohydrate declines (Green, 1984). This is consistent with the fur seal milk collected on shore throughout lactation, where carbohydrate concentrations are low, sodium and potassium levels in the milk are higher than observed for other species, and the volume of milk produced is greater than that at sea (Arnould & Boyd, 1995a). While sodium and potassium levels in the milk are high, a major osmole in milk that regulates milk volume in the fur seal and Tammer wallaby is yet to be identified.

Changes in milk protein during lactation/during a suckling bout

Previous studies in the fur seal reported little variation in total milk protein concentration as lactation progressed (Arnould & Boyd, 1995b) consistent with findings in otariids (Georges et al. 2001). However, a more recent study (Cane et al. 2005) analysing the milk of the Australian fur seal and the Antarctic fur seal (*Arctocephalus gazella*) during the lactation cycle showed no significant change in either the total milk protein or the individual proteins.

Several studies of otariid lactation have reported changes in the concentration of milk components during a suckling period ashore (Costa & Gentry, 1986; Arnould & Boyd, 1995a, b; Goldsworthy & Crowley, 1999; Ochoa-acuna et al. 1999; Georges et al. 2001). Arnould and Boyd (1995b) reported a decline in both milk lipid and protein content during 1–2-d nursing periods of the Antarctic fur seal. Milk protein content declined after 16–24 h ashore, and yet the amount of protein in the milk initially was found to be correlated with the duration of the previous foraging trip (Arnould & Boyd, 1995b). Similar declines in milk protein content for the subantarctic fur seal were reported by Goldsworthy & Crowley (1999) and the Juan Fernandez fur seal, *Arctocephalus philippii* (Ochoa-acuna et al. 1999) after resumption of lactation following arrival back on land.

The β-lactoglobulin genes

Fur seal whey was fractionated by SDSPAGE and β-lg was identified by N-terminal sequencing (Cane et al. 2005).

Donkey P13613, P19647; Cat P33687, P21664, P33688; Dog P33685, S33878; Goat P02756; Buffalo P02755; Dolphin Q7M2T0; Possum Q29146; Wallaby P29614; Kangaroo P11944; Baboon O77511; Platypus, JA Sharp, unpublished observations). (C) Northern blot analysis of milk protein gene expression in the mammary gland of pregnant and lactating on-shore and lactating off-shore fur seals. Total RNA (10 μg) was electrophoresed in 1% agarose gels, blotted to Zeta Probe and hybridized with ^{32}P-labelled cDNA for α-casein and β-lg genes. Lower panel shows ethidium bromide stained gel prior to Northern blot for comparison of RNA loading and integrity. The 18s and 28s RNA bands are indicated.

Screening of cDNA libraries from fur seal lactating on-shore and off-shore mammary glands (Cane et al. unpublished) revealed the presence of two β-lg genes with significant homology to two canine β-lg amino acid sequences (β-lg I, Pervaiz & Brew, 1986; β-lg II, Halliday et al. 1993) and three feline β-lg amino acid sequences (Halliday et al. 1990) (Fig. 1A). Phylogenetic analysis of fur seal β-lg I and II sequences suggests fur seal β-lg II is more closely related to feline β-lg II and groups with horse and donkey β-lg II, while fur seal β-lg I is more divergent (Fig. 1B).

The fur seal mammary gland

The lactation cycle: morphology of the lactating mammary gland during suckling and foraging

Like all mammals, the fur seal mammary gland undergoes an intense period of lobulo-alveolar development during pregnancy and, at lactation, the gland is almost entirely composed of secretory epithelium (Fig. 2A and B). Myoepithelial cells encase the luminal epithelial cells in the ducts, and are in contact with a laminin and collagen IV-rich basement membrane. Surrounding the ductal network, and accounting for >80% of the mammary volume, is a highly compartmentalized stroma. During lactation on shore, the alveoli are engorged with milk containing a large amount of lipid (Fig. 2B). During the mothers' extended foraging trip, the alveoli appear less distended, epithelial cells surrounding the alveoli appear more columnar, and the lipid component within the milk is decreased (Fig. 2C). The reduction of milk protein secretion correlates with decreased milk volume (Arnould & Boyd, 1995b) and histological observations suggest that reduction of milk volume must occur quickly as the mammary gland does not appear full or engorged whilst foraging.

In most mammals during natural weaning, as alveoli fill with milk owing to cessation of suckling, the mammary epithelial cells start to down-regulate milk protein gene expression and the epithelium regresses, and enters involution (Li et al. 1997). This process is characterized by apoptotic cell loss and gland remodelling in readiness for a subsequent pregnancy (Walker et al. 1989; Strange et al. 1992; Lund et al. 1996; Metcalfe et al. 1999). Study of these processes has revealed that involution occurs in two distinct phases (Lund et al. 1996). When experimentally induced by forced weaning the mouse mammary gland initiates the first phase of involution within a few hours of pup removal, and is morphologically characterized by an accumulation of milk in the alveoli and limited apoptosis of epithelial cells (Walker et al. 1989; Strange et al. 1992). This can be reversed up to 1·5 d after weaning when epithelial cell apoptosis starts to dominate the process (Jaggi et al. 1996). Three to five days after weaning the second phase of involution is initiated. It is characterized morphologically by the degradation of the basement membrane, a collapse of alveoli, infiltration of macrophages
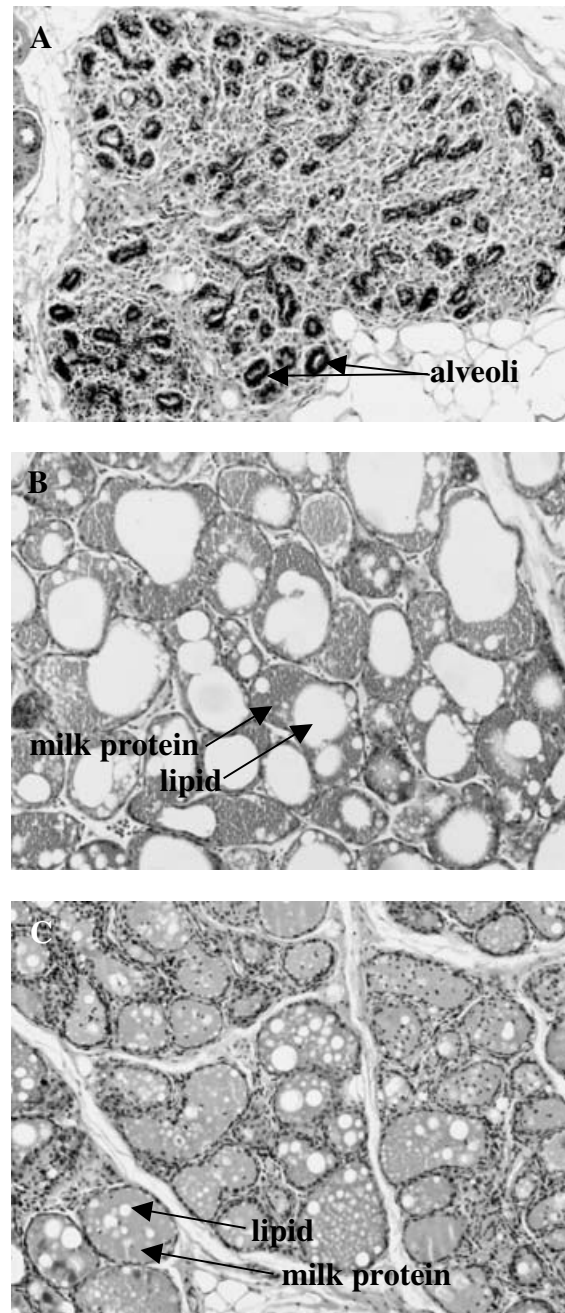

Fig. 2. Histological sections of the mammary gland from (A) pregnant, (B) lactating whilst nursing on shore and (C) lactating whilst foraging at sea fur seals. Sections are stained with Haematoxylin and Eosin. Immature alveoli in the pregnant gland are indicated; lipid (white) and milk protein are indicated in the on shore and off shore mammary glands. Magnification ×100.

and restructuring of the gland to a virgin-like state. During the second phase, apoptosis of epithelial cells continues until 50–80% of the epithelial cells have been cleared from the gland (Walker et al. 1989). Apoptosis associated with involution in the mammary gland of the foraging fur

seal has been analysed using Apotag (Chemicon), and was found to be barely detectable even after extended periods where there is no sucking stimulus, the gland does not regress (Sharp et al. unpublished).

The process by which the lactating fur seal reduces milk production and avoids entering apoptosis whilst foraging is unknown. However it is clear that through the process of evolution and environmental selection, the fur seal appears to have uncoupled the mechanism of milk reduction from involution completely in order to undertake its lactation cycle.

Lactational pause following removal of sucking; down-regulation of milk protein gene expression in off-shore fur seals

The expression of the β-lg and β casein genes is barely detectable in the mammary gland of pregnant fur seals and the level of expression is significantly elevated in the mammary gland of fur seals lactating on-shore (Fig. 1C). However, expression of both genes is also reduced during the foraging trip.

Local factors. A consequence of reduced nursing is that putative factors responsible for regulating apoptosis are retained in the mammary gland. Closure of a single gland of a mouse provoked an accumulation of milk which resulted in changes in gene expression and apoptosis within the closed gland but not of the remaining glands of the same animal (Li et al. 1997; Marti et al. 1997).

Studies in a variety of species indicate that a regulatory mechanism of milk secretion involves a chemical inhibitor (Wilde et al. 1987; Wilde et al. 1989; Wilde et al. 1988; Knight et al. 1994; Peaker et al. 1998). Experiments using *in vitro* models have identified a small whey protein, termed FIL (feedback inhibitor of lactation), that fulfils this role (Wilde et al. 1987; Wilde et al. 1988; Rennison et al. 1993; Blatchford et al. 1998). FIL is synthesized by the secretory epithelial cells of the mammary gland and is secreted into the alveolar lumen along with other milk constituents and acts on the synthesis and secretory pathway by binding a putative receptor on the apical surface of the epithelial cells (Rennison et al. 1993; Blatchford et al. 1998). It is proposed that FIL blocks translation of milk protein transcripts (Rennison et al. 1993) and inhibits secretion of milk constituents. Earlier studies in lactating goats subjected to a reduced frequency of milking for an extended period of time showed longer term effects on mammary cell differentiation and proliferation (Wilde et al. 1995). An inhibitory effect of FIL on milk protein gene expression has been demonstrated in tissue culture models and, interestingly, the response of the cell to reduce milk protein gene transcripts was delayed for 3 d after the commencement of treatment (Blatchford et al. 1998). Therefore, it is conceivable that FIL may play a role in down-regulation of expression of milk protein genes in fur seal the mammary gland during foraging. It may be proposed that the fur seal secretes a molecule with a similar action to FIL that could be present in milk at higher concentrations when the lactating mother goes to sea. This proposal would find support in evidence that the mammary gland of the fur seal has 80% less milk volume whist the fur seal is foraging (Arnould & Boyd, 1995b), and in the histological examination which shows alveoli are not distended with secretory products in lactating mammary tissue on shore. Our preliminary data (K Cane and KR Nicholas, unpublished observations) indicate a FIL-like activity in fractionated fur seal milk. However, the level of inhibitory activity measured was similar to that reported for other species (Blatchford et al. 1998) and did not differ in the milk from fur seals arriving on shore after foraging at sea and after they had been on shore suckling their pups for 1–2 d.

α-Lactalbumin, secreted in milk and involved in the synthesis of lactose, has also recently been implicated in the process of involution (Hakansson et al. 1995; Hakansson et al. 1999; Baltzer et al. 2004). It has been suggested that milk from otariid pinnipeds contains little or no lactose (Schmidt et al. 1971; Dosako et al. 1983; Messer et al. 1988; Urashima et al. 2001), prompting the suggestion that this protein may be absent or cannot function correctly to produce lactose in the fur seal. Recent studies in our laboratory suggest these fur seals do secrete a modified α-lactalbumin (C Reich, JA Sharp, JPY Arnould and KR Nicholas, unpublished observations). However, it remains to be determined whether this α-lactalbumin has any capacity to stimulate apoptosis. It is interesting to speculate that the absence of biologically active α-lactalbumin in milk may be consistent with the absence of apoptosis in the mammary gland of lactating fur seals during foraging and that loss of this protein has provided evolutionary pressure to alter the lactational strategy of the Otarrid family of seals.

Systemic factors. Several studies in mice have shown that both systemic and local signals generated by the accumulation of milk regulate the mechanism of milk reduction and the involution process (Quarrie et al. 1995; Travers et al. 1996; Li et al. 1997; Peaker et al. 1998). One scenario speculates that, after weaning, the systemic down-regulation of either prolactin or glucocorticoid levels result in the inhibition of intracellular signalling cascades (Travers et al. 1996; Hennighausen et al. 1997). This is supported by findings that show prolactin treatment following litter removal in mice delays mammary apoptosis (Feng et al. 1995) and exogenously administered glucocorticoids can suppress mammary apoptosis when nursing ceases (Feng et al. 1995; Lund et al. 1996). Plasma prolactin in the Antarctic fur seal, measured using a human prolactin standard, was elevated from 1–2 d before parturition and peaked during the perinatal period at 0–3 d post partum. It then declined slowly throughout the remainder of the perinatal period and remained at a low
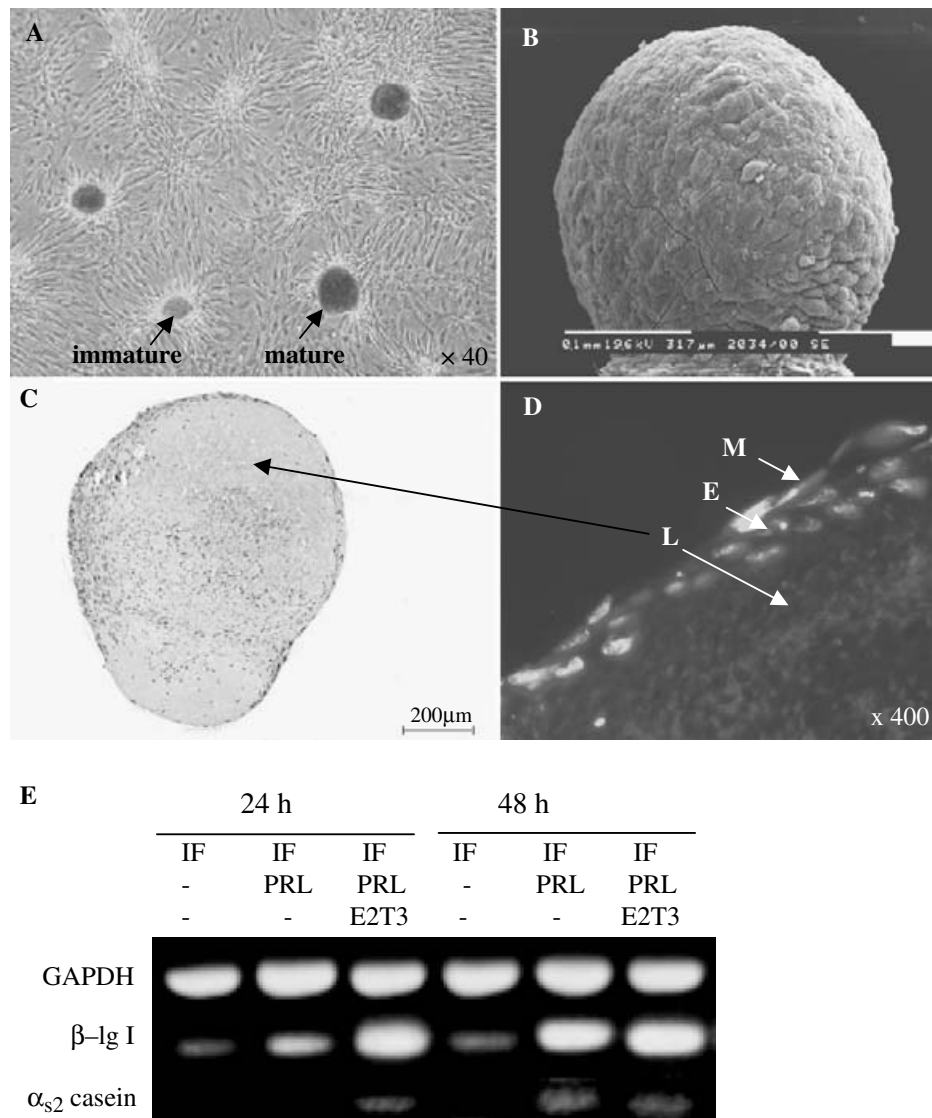
Fig. 3. Development of fur seal mammospheres. (A) phase contrast images of mammospheres on tissue culture treated plastic showing immature and mature mammospheres; (B) electron micrograph of whole mounted mammosphere; (C) Haematoxylin and eosin stained 5-μm section (5 μm) of a mammosphere showing central lumen (L). (D) a section (5 μm) of a mammosphere stained with DAPI showing epithelial cell organization. Cellular types are shown by arrows (M) myoepithelial cell, (E) epithelial cells, and the lumen is indicated by (L). (E) Prolactin-responsive expression of the β-lactoglobulin and α_{-s2} casein gene in fur seal mammospheres. RT-PCR analysis of total RNA from mammospheres was used for detection of expression of the milk protein genes, BLG-I and α_{s2}-casein after 24-h and 48-h exposure to different combinations of insulin (I; 1 μg/ml), cortisol (F; 50 ng/.ml), prolactin (PRL; 1 μg/ml), oestrogen (E2; 25 pg/ml) and thyroid hormone (T3; 6·5 ng/ml).

level. There was also no significant change in the hormone levels between the lactating mammary gland of nursing fur seals (Boyd, 1991). Therefore, if prolactin levels were either maintained or increased in the foraging fur seal, a prolactin survival cascade may be protecting the epithelial cell of the mammary gland in the foraging fur seal and preventing the gland from entering involution while a local mechanism inhibits milk synthesis and secretion.

A potential role for glucocorticoids and IGF, as possible mediators of survival signals in the mammary gland (LeRoith et al. 1995; Lund et al. 1996; Farrelly et al. 1999), has not been examined in the fur seal, but may provide a mechanistic role in protecting epithelial cells of the mammary gland from involution (Feng et al. 1995; Hadsell et al. 1996) as milk production decreases.

Mechanical stress. Local signals resulting from engorgement, causing stress between the interaction of the extracellular matrix (ECM) and alveoli epithelial cells, may initiate new and independent signalling cascades that activate the apoptotic programme during the first phase of involution (Boudreau et al. 1995; Clark & Brugge,

1995). To overcome this, it is likely that the fur seal reduces its milk production while going to sea to forage, to ensure the alveoli are not engorged, so limiting the stress on alveoli.

It is postulated that if the BM becomes stretched and alters the molecular interactions with adhesion receptors, it may lead to reduced ligand-binding interacting sites (Banes et al. 1995). For example, the levels of ligand-bound $\beta 1$ integrin are significantly decreased during the transition from lactation to involution in mice (McMahon et al. 2004) and direct attachment of epithelial cells to the ECM occurs through basally located integrins (Alford & Taylor-Papadimitriou, 1996; Weaver et al. 1997). The affinity modulation of integrin activity and, therefore, a potential inability to respond to survival signals from the basement membrane may contribute to the induction of apoptosis at the onset of involution. We have found evidence that $\beta 1$ integrin is up-regulated in the foraging fur seal mammary gland compared with the on-shore nursing mammary gland, and we predict this would assist in counteracting any effects of the loss of the $\beta 1$ integrin/epithelial cell interaction if the gland was under any form of mechanical stress during foraging (JA Sharp, unpublished observations).

Indeed, a candidate mechanism for avoiding alveoli collapse and cell death is up-regulation of ECM components thus avoiding degradation of the ECM, preventing the transduction of apoptotic signals (Blatchford et al. 1999). In this context, it is interesting that we have found that fur seal mammary epithelial cells when grown in culture have the unique capacity to secrete significant amounts of ECM, which in turn leads to formation of hormone-responsive mammospheres.

Fur seal *in vitro* mammary model

Fur seal mammospheres; an in vitro model to study mammary function

The study of mammary gland differentiation and lactation *in vivo* is difficult in species such as the fur seal where access to mammary tissue is limited. Mammary cells cultured in 3D to form alveoli-like mammospheres (Barcellos-Hoff et al. 1989; Blatchford et al. 1999) offer an attractive system in which to identify local factors that control the susceptibility of lactating mammary epithelial cells to apoptosis. We have prepared a mammary epithelial cell-enriched fraction from the gland of a mid-pregnant fur seal and these cells initially grow as a mono-layer when introduced to either plastic or a suspended pliable membrane. However, fur seal mammary cells secrete an ECM which subsequently initiates formation of mammospheres (Fig. 3). In contrast, mammary cells of other species such as the cow, human and mouse do not secrete their own matrix and require exogenous Matrigel for mammosphere formation (Li et al. 1987; Barcellos-Hoff et al. 1989; Stoker et al. 1990; Ackland et al. 2001). Over a

period of 14 d fur seal mammospheres undergo cavitation to form a lumen, presumably by initiating regulated apoptosis of the cells within the structure (Blatchford et al. 1999) leaving a thin layer of epithelial cell on the surface to resemble the normal mammary alveolus (Fig. 3C and D). The basolateral polarity of the cells is maintained within mammospheres, and as for mammospheres derived from other species, fur seal mammospheres are capable of mammary gland-specific function such as expression of the βlg-I and α_{s2} casein genes in response to lactogenic hormones (Fig. 3E). The use of the mammosphere model will allow characterization of local factors to elucidate further the mechanism of uncoupling milk production and involution in the lactating mammary gland of the fur seal.

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