The Tammar Wallaby and Fur Seal: Models to Examine Local Control of Lactation¹

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ABSTRACT

Mammary development and function are regulated by systemic endocrine factors and by autocrine mechanisms intrinsic to the mammary gland, both of which act concurrently. The composition of milk includes nutritional and developmental factors that are crucial to the development of the suckled young, but it is becoming increasingly apparent that milk also has a role in regulating mammary function. This review examines the option of exploiting the comparative biology of species with extreme adaptation to lactation to examine regulatory mechanisms that are present but not readily apparent in other laboratory and livestock species. The tammar wallaby has adopted a reproductive strategy that includes a short gestation (26 d), birth of an immature young, and a relatively long lactation (300 d). The composition of milk changes progressively during the lactation cycle, and this is controlled by the mother and not the sucking pattern of the young. Furthermore, the tammar can practice concurrent asynchronous lactation; the mother provides a concentrated milk high in protein and fat for an older animal that is out of the pouch and a dilute milk low in fat and protein but high in carbohydrates from an adjacent mammary gland for a newborn pouch young. This phenomenon suggests that the mammary gland is controlled locally. The second study species, the Cape fur seal, has a lactation characterized by a repeated cycle of long at-sea foraging trips (up to 28 d) alternating with short suckling periods of 2 to 3 d ashore. Lactation almost ceases while the seal is off shore, but the mammary gland does not progress to apoptosis and involution, most likely because of local control of the mammary gland. Our studies have exploited the comparative biology of these models to investigate how mammary function is regulated by endocrine factors, and particularly by milk. This review reports

3 major findings using these model animals. First, the mammary epithelial cell has an extraordinary intrinsic capacity for survival in our culture model, and the path to either function or death by apoptosis is actively driven. The second outcome is that the route to apoptosis is most likely regulated by specific milk factors. Finally, whey acidic protein, a major milk protein in some species, may play a role in normal mammary development, but that role in vivo may be limited to marsupials. Evolutionary pressure has led to changes in the structure of the protein with an accompanying change in function. Therefore, we propose that a loss of function of this protein in eutherians may relate to a reproductive strategy that is less dependent on lactation.

Key words: lactation, local control, mammary gland

INTRODUCTION

Our understanding of the endocrine regulation of lactation in most species is advanced, but it is now clear that local regulation of mammary function is equally important and acts concomitantly with endocrine stimuli. Animal models with extreme adaptation to lactation can prove useful for increasing our understanding of the latter process by revealing mechanisms that are present but not readily apparent in many eutherian animals. The lactation cycle is common to all mammals, although marsupials and some pinnipeds have evolved a reproductive strategy distinct from most eutherians. The latter have a long gestation relative to their lactation period, whereas reproduction in marsupials such as the tammar wallaby (*Macropus eugenii*) is characterized by a short gestation followed by a long lactation, and secretion of all the major milk constituents changes progressively during lactation (Nicholas, 1988; Tynedale-Biscoe and Janssens, 1988). The lactation cycle of the Cape fur seal (*Arctocephalus pusillus pusillus*) is composed of maternal foraging and infant nursing periods that are spatially and temporally separate (Boyd, 1998). Lactating mothers suckle offspring over a period of many months, and females alternate between short periods ashore suckling their young and longer periods

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Figure 1. The lactation cycle of the tamarin wallaby. (A) Development of the pouch young from d 6 to 220 of age. (B) The lactation cycle in the tamarin has been divided into 4 phases, characterized by changes in milk composition and the sucking pattern of the pouch young. (C) Expression of the major milk protein genes during the lactation cycle. The α -CN, β -CN, α -LA, and β -LG genes are induced at parturition and expression remains elevated for the entire lactation. The genes for ELP (early-lactation protein), WAP (whey acidic protein), and the LLP (late-lactation proteins A and B) are expressed only for specific phases of the lactation cycle.

of up to 4 wk foraging at sea (Gentry and Holt, 1986). These animal models provide new opportunities to examine local regulation of mammary function, and particularly the potential role of milk in this process.

The Tamarin Wallaby

Lactation in the tamarin has been divided into phases that are defined by the composition of the milk and the apparent sucking pattern of the young (Figure 1; Nicholas et al., 1995, 1997). Phase 1 is a 26.5-d pregnancy, and the subsequent 200 d of phase 2 is characterized by lactogenesis and the secretion of small volumes of dilute milk high in carbohydrates and low in fat and protein. The pouch young remains attached to the teat for approximately the first 100 d (phase 2A), after which it relinquishes the teat, sucking less frequently while

remaining permanently in the pouch (phase 2B) for an additional 100 d. Phase 3 of lactation (200 to 330 d) is characterized by a large increase in milk production, and the composition of milk changes to include elevated levels of protein and lipids and low levels of carbohydrates (Figure 1; Nicholas et al., 1995, 1997).

Two temporally different patterns of milk protein gene expression appear during the lactation cycle: One group of genes is induced to high levels around parturition and expressed throughout lactation, and a second group of genes is expressed only during specific phases of lactation (Figure 1C; Simpson and Nicholas, 2002). For example, the genes for the whey proteins β -LG and α -LA, and the α -CN and β -CN genes are induced coordinately and independently of the sucking stimulus at parturition and are expressed for the duration of lactation. In contrast, the early lactation protein gene

Figure 2. Concurrent asynchronous lactation. The pouch has been retracted to expose the 4 mammary glands. A 6-d-old young is attached to a teat from a mammary gland secreting phase 2A milk. An older animal at approximately 275 d of age has vacated the pouch and sucks from the elongated teat, which provides phase 3 milk from the enlarged mammary gland. The remaining 2 teats are from quiescent mammary glands.

(*Elp*) is expressed at very high levels in phase 2A, the whey acidic protein gene (*Wap*) most highly in phase 2B, and 2 genes (*Llp-A*, *Llp-B*) coding for outlier lipocalin proteins, referred to as late-lactation proteins (**LLP**) A and B, are highly expressed in phase 3.

The lactating tammar regulates these changes in milk composition, which in turn determines the rate of growth and development of the pouch young (Trott et al., 2003). If a pouch young from an early stage of lactation is transferred to the mammary gland of a tammar at a later stage of lactation, the changed sucking pattern does not lead to any changes in the timing of secretion of milk components, suggesting that the mother regulates this process and that it is not cued by the pouch young. Furthermore, the tammar can perform asynchronous concurrent lactation, whereby the mother concomitantly provides milk for an older animal out of the pouch and at heel, and milk of an entirely different composition from an adjacent mammary gland for a newborn pouch young (Figure 2; Nicholas, 1988). Assuming that both mammary glands are exposed to the same systemic milieu of hormones, this observation is consistent with mammary function being regulated by factors or processes intrinsic to the gland.

Cape Fur Seal

The Cape fur seal suckles its pup for 10 mo (Gentry and Holt, 1986), and lactation is characterized by alter-

nate periods of several days ashore suckling the young and extended periods at sea (Figure 3; Bonner, 1984; Oftedal et al., 1987; Trillmich, 1996). Foraging trips are variable but can extend to 25 d (Gamel et al., 2005). Nursing periods are usually short, lasting only 1 to 3 d before the mother's body reserves have again been depleted and she returns to forage.

During the mother's time at sea, the mammary gland does not involute but produces less milk compared with that of the onshore lactating female. For example, milk production in Antarctic fur seals (*Arctocephalus gazella*) has been shown to continue while the female is foraging at sea but at only 19% the rate of production on land (Arnould and Boyd, 1995). Milk protein gene expression also decreases during the foraging trip: The β -CN, α_{S2} -CN, and β -LG genes are all down-regulated in the mammary gland of the foraging Cape fur seal (Cane, 2005; Sharp et al., 2006).

SCOPE AND APPROACH

This review focuses on the potential role of milk in regulating mammary development and function. First, we consider experiments focusing on milk as a potential factor directing remodeling of the mammary gland during involution. Milk accumulates in the alveoli and signals involution, either by chemical factors in the milk or by factors responding to physical distension of the mammary alveolus. A comparison between the tammar and the seal, the latter being a species in which the mammary gland does not involute despite cessation of sucking while the animal is foraging in the ocean, may provide new insights into the chemical signaling of the mammary gland. Second, we describe experiments that explore a potential role for whey acidic protein (**WAP**) on the development of the mammary gland in the tammar. Surprisingly, we know little about the function of many of the major milk proteins, but the tammar may provide new opportunities to examine the potential role of this protein in the development of either the mammary gland or the suckled young.

THE TAMMAR: A ROLE FOR MILK IN THE CONTROL OF MAMMARY FUNCTION

The expression of milk protein genes is regulated concurrently by systemic endocrine factors, by paracrine factors such as the extracellular matrix, and by autocrine factors secreted in the milk. Previous studies using a tammar mammary explant culture model have shown that different combinations of insulin, cortisol, and prolactin are required for expression of the α - and β -CN and whey protein genes, including α -LA and β -LG (Simpson and Nicholas, 2002). Interestingly, tammar

Figure 3. The “foraging” lactation strategy of the fur seal. The pregnant female arrives at shore to give birth and remains with the pup for approximately 1 wk. For the remainder of lactation, females alternate trips to sea with short trips ashore to suckle their pup. Reprinted from *Current Topics in Developmental Biology*, 72, G. P. Schatten, ser. ed., J. A. Sharp, K. N. Cane, C. Lefevre, J. P. Y. Arnold, and K. R. Nicholas, “Fur Seal Adaptations to Lactation: Insights into Mammary Gland Function,” pages 276–308, Academic Press, New York. Copyright 2006, with permission from Elsevier.

explants from late-pregnant tammar can be induced to express the *Wap* gene with insulin, cortisol, prolactin, thyroid hormone, and estrogen (Simpson et al., 2000). Therefore, the inhibition normally observed in vivo during phase 2A and the subsequent induction of *Wap* gene expression at approximately 100 d postpartum may be hormonally regulated. Alternatively, the *Llp* genes could be down-regulated in mammary explants from phase 3 tammar and then restimulated with insulin, cortisol, and prolactin, but expression of these genes could not be induced in mammary explants from pregnant tammar with any hormone combination tested (Trott et al., 2002). Either the appropriate hormonal milieu was not used or the tissue required additional factors to express these genes. These conclusions are supported by an earlier study showing that constructs with up to 1.8 kb of the *Llp-A* gene promoter did not express a reporter gene after transfection into Chinese hamster ovary (CHO) cells incubated with insulin, cortisol, and prolactin. In addition, the same construct was not expressed in lactating transgenic mice (Trott et al., 2002).

There is increasing evidence to suggest that milk plays an important role in regulating mammary epithelial function and survival, and this is particularly evident during involution. Apoptosis was induced preferentially in the sealed teats of lactating mice while the litter suckled successfully on the remaining teats, which indicates that cell death is stimulated by an intramammary mechanism sensitive to milk accumulation (Quarrie et al., 1995). A protein known as the feedback inhibitor of lactation (**FIL**) has been suggested as a candidate

and is secreted in the milk of the tammar (Hendry et al. 1998) and other species. It acts specifically through interaction with the apical surface of the mammary epithelial cell to reduce secretion (Wilde et al., 1995).

More recent studies using the tammar mammary explant culture model to examine the process of involution have confirmed the likely role of milk (and putative autocrine factors) for controlling mammary function during involution. Mammary explants from pregnant tammar were cultured for 3 d with insulin, cortisol, and prolactin to induce milk protein gene expression. To mimic involution, all hormones were subsequently removed from the culture medium for 10 d to down-regulate expression of the milk protein genes (Figure 4). Surprisingly, the explants retained the same level of response during a subsequent challenge with lactogenic hormones. Previous studies have shown that secretion of milk proteins from tammar mammary explants is limited, but it is unlikely that milk constituents accumulate to elevated concentrations (Nicholas and Tynedale-Biscoe, 1985). The maintenance of epithelial cell viability and hormone responsiveness in explants cultured in the absence of hormones suggests a more active mechanism, such as the accumulation of local factors in the milk being the primary stimulus for apoptosis of mammary epithelial cells in the tammar wallaby mammary gland. However, the primary outcome of these studies is evidence for the extraordinary capacity for survival and maintenance of hormone responsiveness by tammar mammary epithelial cells cultured in a chemically defined medium with no exogenous hormones and growth factors.

Figure 4. Northern blot analysis of β -LG, α -CN, and α -LA gene expression in mammary explants. Gene expression is shown in mammary tissue from pregnant tammars at d 24 prior to culture (t_0), explants cultured in media with insulin, cortisol, and prolactin (IFP) for 3 d, explants subsequently cultured in the absence of hormones (NH) for 10 d, and following the reintroduction of IFP for 3 d. The length of culture in days is shown by the subscript. Total RNA (10 μ g, lower panel) was assayed by Northern blot analysis using [α - 32 P]dCTP-labeled cDNA probes for the β -LG, α -CN, and α -LA genes (upper panels). Arrows indicate transcript size in nucleotides and RNA ribosomal bands.

THE CAPE FUR SEAL: MILK PROTEIN GENE EXPRESSION IN THE MAMMARY GLAND DURING SUCKLING AND FORAGING

During lactation of the Cape fur seal on shore, the mammary alveoli are engorged with milk containing a large amount of lipids (Sharp et al., 2006). During the mother's extended foraging trip, the alveoli appear less distended, epithelial cells surrounding the alveoli ap-

pear more columnar, and the lipid component is decreased within the milk. Expression of the β -CN gene is barely detectable in the mammary gland of pregnant seals, and the level of expression is significantly elevated in the mammary gland of seals lactating on shore (Figure 5). However, expression of this gene is also reduced during the foraging trip. High sequence conservation between the Cape fur seal and dog, 95% similarity at the DNA level (Sharp et al., 2006), permits a significant detection rate of measurable hybridization signals between seal cDNA and the Affymetrix canine microarray. Cluster analysis of expression profiles from these data has revealed that the overall expression profile of the lactating mammary gland of the foraging Cape fur seal is more closely related to the profile of pregnant nonlactating animals (placental gestation) than the profile obtained from onshore lactating animals (in embryonic diapause) (Figure 5). This result suggests that the interruption of lactation in foraging animals involves a major deprogramming of mammary gland gene expression.

During natural weaning in most mammals, as alveoli fill with milk because suckling ceases, the mammary epithelial cells begin to decrease milk protein gene expression and the epithelium regresses and enters involution (Li et al., 1997). This process is characterized by apoptotic cell loss and mammary gland remodeling (Strange et al., 1992; Lund et al., 1996; Metcalfe et al., 1999). Apoptosis associated with involution in the mammary gland of the foraging seal has been analyzed and found to be barely detectable; the gland does not regress even after extended periods when there is no sucking stimulus (J. A. Sharp, K. R. Nicholas; unpublished manuscript). The process by which the lactating seal reduces milk production and avoids entering apoptosis while foraging is unknown. However, it is clear that through the process of evolution and environmental selection, the seal appears to have uncoupled the mechanism of milk reduction from involution to undertake its lactation cycle.

A consequence of reduced nursing is that putative factors responsible for regulating apoptosis are retained in the mammary gland. Sealing of a single mammary teat of a mouse has been shown to induce an accumulation of milk, resulting in changes in mammary gene expression and apoptosis within the sealed gland but not in the remaining glands of the same animal (Li et al., 1997; Marti et al., 1997). Studies in lactating animals from a variety of species indicate that a regulatory mechanism of milk secretion may involve a chemical inhibitor (Knight et al., 1994; Wilde et al., 1995; Peaker et al., 1998). As mentioned previously, experiments have identified a small whey protein, termed FIL (Wilde et al., 1995), that is synthesized by the secretory epithe-

Figure 5. Histological sections of the mammary gland from (A) the lactating Cape fur seal while nursing on shore and (B) the lactating Cape fur seal while foraging at sea. Sections are stained with hematoxylin and eosin. Immature alveoli in the pregnant gland are indicated: lipids (white) and milk proteins (pink) are indicated in the onshore and offshore lactating mammary glands. Magnification $\times 100$. Milk protein gene expression (C): β -CN expression during the Cape fur seal lactation cycle. Analysis of expression using canine Affymetrix chips hybridized to cDNA probes generated from RNA from the pregnant (placental gestation and nonlactating, $n = 2$) and lactating Cape fur seal on shore ($n = 2$) and at sea ($n = 1$; animals in embryonic diapause). (D) Cluster analysis of gene expression profiles from the Cape fur seal mammary gland during different stages of lactation. A total of 1,020 Cape fur seal mammary messenger RNA (mRNA) transcripts were identified with expression levels above an intensity of 250 in any sample type. Hierarchical clustering was conducted using Euclidean distance. Pregnant (placental gestation and nonlactating) and onshore lactating (in embryonic diapause) data represent an average of 2 animals. Offshore data represent a single sample. Reprinted from *Current Topics in Developmental Biology*, 72, G. P. Schatten, ser. ed., J. A. Sharp, K. N. Cane, C. Lefevre, J. P. Y. Arnold, and K. R. Nicholas, "Fur Seal Adaptations to Lactation: Insights into Mammary Gland Function," pages 276–308, Academic Press, New York. Copyright 2006, with permission from Elsevier.

lial cells of the mammary gland and secreted into the alveolar lumen along with other milk constituents. It has been proposed that FIL 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). Furthermore, FIL may block the translation of milk protein transcripts (Rennison et al., 1993) and inhibit the secretion of milk constituents. Preliminary data (Cane, 2005) have demonstrated a FIL-like activity in fractionated seal milk, but the level of inhibitory activity measured was similar to that reported for other species (Blatchford et al., 1998). In addition, the activity did not differ in milk collected from seals arriving on shore after foraging at sea and after they had been on shore suckling their pups for 1 to 2 d. Generally, studies to better define the role of FIL have been hampered by a lack of characterization of this molecule and by a limited understanding of the molecular mechanisms of its action on mammary epithelial cells.

The α -LA protein is required for lactose synthesis and is secreted in the milk (Schmidt et al., 1971). Studies have suggested that this protein may be implicated in the process of involution (Hakansson et al., 1995, 1999; Baltzer et al., 2004). Milk from otariid pinnipeds contains little or no lactose (Urashima et al., 2001), suggesting that this protein may be absent in these species. Recent studies in our laboratory have suggested that these seals may secrete a modified α -LA (C. Reich, J. A. Sharp, Cooperative Research Centre (CRC) for Innovative Dairy Products, Department of Zoology, University of Melbourne, Australia; C. Lefevre, CRC for Innovative Dairy Products, Department of Zoology, University of Melbourne, Australia, and Victorian Bioinformatics Consortium, Monash University, Clayton, Victoria, Australia; J. P. Y. Arnould, School of Life and Environmental Science, Deakin University, Australia, and K. R. Nicholas, CRC for Innovative Dairy Products, Department of Zoology, University of Melbourne, Australia, unpublished manuscript). However, it remains to be determined whether this α -LA has any capacity to stimulate apoptosis. It is interesting to speculate that the absence of biologically active α -LA in milk may be consistent with the absence of apoptosis in the mammary gland of lactating seals during foraging and that loss of this protein has provided an opportunity to alter the lactational strategy of the Otariid family of seals.

Local signals resulting from engorgement, causing stress in the interaction between the extracellular matrix and alveoli epithelial cells, may initiate new and independent signaling cascades that activate the apoptotic program during the first phase of involution (Boudreau et al., 1995; Clark and Brugge, 1995). To over-

come this, it is likely that the seal reduces its milk production while going to sea to forage to ensure that the alveoli are not engorged, limiting the stress within the alveoli. It is postulated that if the basement membrane becomes stretched and alters the molecular interactions with adhesion receptors, it leads to reduced ligand-binding sites (Banes et al., 1995). The levels of ligand-bound β 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 extracellular matrix occurs through basally located integrins (Alford and Taylor-Papadimitriou, 1996; Weaver et al., 1997). The modulation of integrin activity, and therefore the potential inability to respond to survival signals from the basement membrane, may contribute to the induction of apoptosis at the onset of involution.

WAP: A ROLE IN LOCAL REGULATION OF MAMMARY DEVELOPMENT IN THE TAMMAR?

Identifying the primary function for many milk proteins, including WAP, has proved challenging. A major whey protein gene, *Wap*, is expressed throughout lactation in many eutherian species, including the rat, mouse, rabbit, camel, and pig (see Simpson et al., 2000; Simpson and Nicholas, 2002). In addition to the tammar, WAP is asynchronously secreted in the milk of other marsupials, including the red kangaroo (Nicholas et al., 2001), opossum (Demmer et al., 2001), and stripe-faced dunnart (DeLeo et al., 2006). The AA sequence of WAP from the 2 monotremes, the platypus and echidna, has been reported (Simpson et al., 2000), but the pattern of secretion in these species has yet to be established.

Alignment of the AA sequence of WAP proteins from marsupial and eutherian species shows limited sequence identity (Simpson et al., 2000). However, these proteins are recognized by the presence of the WAP motif (KXGXCP) and a domain structure known as the 4-disulfide core domain, which consists of 8 Cys residues (Ranganathan et al., 1999). The WAP secreted in the milk of eutherians have two 4-disulfide core domains (Simpson and Nicholas, 2002), whereas WAP in all marsupials studied to date have 3 domains (Simpson et al., 2000; Demmer et al., 2001; DeLeo et al., 2006). Consequently, the 5 exons of the marsupial *Wap* genes contrast with the eutherian *Wap* genes, which have only 4 exons (DeLeo et al., 2006). The significance of the loss of the third domain in eutherians remains unclear, particularly because a biological function for milk WAP remains to be established.

The phase-specific expression of the tammar *Wap* gene between 100 and 200 d of lactation may provide

Figure 6. (A) Proliferation of wallaby mammary epithelial cells (Wall-MEC) cells cultured in the presence and absence of tammar WAP (tWAP). Results shown are after d 3 of treatment with (+) and without (–) tWAP. Cell growth was analyzed using methods described by Skehan et al. (1990). The vertical bars indicate SEM ($n = 3$, $*P < 0.05$). (B) Expression of the cyclin D1 gene in Wall-MEC cells grown in the presence and absence of tammar WAP (d 3 posttreatment). Expression of the cyclin D1 and GAPDH genes was determined by reverse transcription PCR analyses. The relative expression levels of cyclin D1 and CDK-4 genes in Wall-MEC were quantified by National Institutes of Health Image software by analyzing reverse transcription PCR products. The results show the level of cyclin D1 expression as a proportion of the expression of the housekeeping gene GAPDH.

new opportunities to explore a function for the corresponding protein, both for regulating lactation and for development of the young. Recent studies have shown that mouse WAP added to culture medium of mouse HC-11 cells is antiproliferative, possibly acting by an autocrine or paracrine mechanism (Nukumi et al.,

2004). This is consistent with reports showing that overexpression of WAP in transgenic mice inhibits development of the mammary gland and secretion of milk (Burdon et al., 1991).

Recent studies in our laboratory have used in vitro models to examine the effect of tammar WAP on the proliferation of tammar mammary epithelial cells. Tammar WAP has been synthesized in vitro and added to culture medium. In contrast to the inhibitory action of mouse WAP on the proliferation of HC-11 cells, preliminary results suggest that tammar WAP stimulates the proliferation of mammary epithelial cells and increases expression of the cell cycle gene cyclin D1 (Figure 6). Earlier studies have shown that DNA synthesis in mammary tissue is higher in phase 2 than in phase 3 (Nicholas, 1988), which is consistent with a potential role of tammar WAP in this process. Therefore, it is conceivable that tammar WAP plays a role in development of the tammar mammary gland and may be important to support the reproductive strategy of marsupials and monotremes. Interestingly, studies using a *Wap* gene knockout mouse have shown that mammary development is normal. However, the pups had limited development at the later stages of lactation (Triplett et al., 2005). Because tammar WAP is a major milk protein that is most highly secreted during the middle third of lactation, at a time when the pouch young diet consists of only milk, it could be speculated that this protein plays a specific role in the development of both the mammary gland and the suckled young. The *Wap* gene in the human, cow, ewe, and goat is predicted to be a pseudogene (see Hajjoui et al., 2006). It is tempting to suggest that WAP function is predominantly found only in marsupials and that the activity has been lost because of a loss of evolutionary pressure on this protein, which relates to changes in the reproductive strategy of eutherians. It is therefore conceivable that the marsupial may be a more appropriate model to explore the potential of other major milk proteins such as β -LG, which, like *Wap*, is not expressed in all eutherians. The value of these experimental models for increased understanding of the local regulation of mammary function in mainstream laboratory and livestock species will be increasingly attractive.

CONCLUDING REMARKS

This paper describes the exploration of 2 animal models for the study of lactation control and the comparative analysis of mammalian lactation evolution. Our comparative approach illustrates how, by using animal models with extreme adaptations, it is possible to extend beyond the simple observation of conservation of function and reveal subtle functional changes in specific

genes. For example, structural changes such as the occurrence of an extra domain in marsupial WAP potentially lead to functional changes. On the other hand, *Wap* has been lost altogether in some eutherians, including humans. In addition, marsupial LLP have as yet no characterized homologs in eutherians. Similarly, LLP have been lost during eutherian evolution. Alternatively, they have evolved in the marsupial from an as yet uncharacterized ancestral gene. In a similar way, low lactose production in seal milk may be related to a reduced production of α -LA. This situation might have provided an opportunity for the evolution of the unusual lactation pattern of the Cape fur seal and related otariids. These observations raise many questions and furnish interesting new hypotheses for the evolution of lactation control.

In the future, comparative analyses and functional genomics of mammary gene expression will provide a refined view of the full spectrum of changes in gene expression in the lactation strategies of these animal models. These experiments will assist the study of the association of mammary genes into functional and control networks. Moreover, the potential role of milk as vector of functional information between the mother and young suggests that functional networks extend beyond local control of the mammary gland alone and into the physiology of the young, such as the role of milk components in early development of the marsupial young.

REFERENCES

- Alford, D., and J. Taylor-Papadimitriou. 1996. Cell adhesion molecules in the normal and cancerous mammary gland. *J. Mammary Gland Biol. Neoplasia* 1:207–218.
- Arnould, J. P., and L. L. Boyd. 1995. Temporal patterns of milk production in Atlantic fur seals (*Arctocephalus gazella*). *J. Zool.* 237:1–12.
- Baltzer, A., C. Svanborg, and R. Jaggi. 2004. Apoptotic cell death in the lactating mammary gland is enhanced by a folding variant of α -lactalbumin. *Cell. Mol. Life Sci.* 61:1221–1228.
- Banes, A. J., M. Tsuzaki, J. Yamamoto, T. Fischer, B. Brigman, T. Brown, and L. Miller. 1995. Mechanoreception at the cellular level: The detection, interpretation, and diversity of responses to mechanical signals. *Biochem. Cell Biol.* 73:349–365.
- Blatchford, D. R., K. A. Hendry, and C. J. Wilde. 1998. Autocrine regulation of protein secretion in mouse mammary epithelial cells. *Biochem. Biophys. Res. Commun.* 248:761–766.
- Bonner, W. N. 1984. Lactation strategies of pinnipeds: Problems for a marine mammalian group. *Symp. Zool. Soc. London* 51:253–272.
- Boudreau, N., C. J. Simpson, Z. Werb, and M. J. Bissell. 1995. Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. *Science* 267:891–893.
- Boyd, I. L. 1988. Time and energy constraints in pinniped lactation. *Am. Nat.* 152:717–728.
- Burdon, T., R. J. Wall, A. Shamay, G. H. Smith, and L. Hennighausen. 1991. Over-expression of an endogenous milk protein gene in transgenic mice is associated with impaired mammary alveolar development and a milchlos phenotype. *Mech. Dev.* 36:67–74.
- Cane, K. N. 2005. The physiological and molecular regulation of lactation in fur seals. PhD Thesis. University of Melbourne, Melbourne, Australia.
- Clark, E. A., and J. S. Brugge. 1995. Integrins and signal transduction pathways: The road taken. *Science* 268:233–239.
- De Leo, A., C. Lefevre, D. Topcic, E. Pharo, J. F. Cheng, P. Frappell, M. Westerman, J. Graves, and K. Nicholas. 2006. Isolation and characterization of two whey protein genes in the Australian dasyurid marsupial the stripe-faced dunnart (*Sminthopsis macroura*). *Cytogenet. Genome Res.* 115:62–69.
- Demmer, J., S. J. Stasiuk, M. R. Grigor, K. J. Simpson, and K. R. Nicholas. 2001. Differential expression of the whey acidic protein during lactation in the brushtail possum (*Trichosurus vulpecula*). *Biochim. Biophys. Acta* 1522:187–194.
- Gamel, C. M., R. W. Davis, J. H. David, M. A. Meer, and E. Brandon. 2005. Reproductive energetics and female attendance patterns of Cape fur seals (*Arctocephalus pusillus pusillus*) during early lactation. *Amer. Mid. Nat.* 153:152–170.
- Gentry, R. L., and J. R. Holt. 1986. Attendance behaviour of northern fur seals. Pages 41–60 in *Fur Seals: Maternal Strategies on Land and at Sea*. R. L. Gentry and R. L. Kooyman, ed. Princeton University Press, Princeton, NJ.
- Hajjoui, S., S. Rival-Gervier, H. Hayes, S. Floriot, A. Eggan, F. Piumi, P. Chardon, L. M. Houdebine, and D. Thepot. 2006. Ruminants genome no longer contains Whey Acidic Protein gene but only a pseudogene. *Gene* 370:104–112.
- Hakansson, A., J. Andreasson, B. Zhivotovsky, D. Karpman, S. Orrenius, and C. Svanborg. 1999. Multimeric α -lactalbumin from human milk induces apoptosis through a direct effect on cell nuclei. *Exp. Cell Res.* 246:451–460.
- Hakansson, A., B. Zhivotovsky, S. Orrenius, H. Sabharwal, and C. Svanborg. 1995. Apoptosis induced by a human milk protein. *Proc. Natl. Acad. Sci. USA* 92:8064–8068.
- Hendry, K. A., K. J. Simpson, K. R. Nicholas, and C. J. Wilde. 1998. Autocrine inhibition of milk secretion in the lactating tammar wallaby (*Macropus eugenii*). *J. Mol. Endocrinol.* 21:169–177.
- Li, M., X. Liu, G. Robinson, U. Bar-Peled, K. U. Wagner, W. S. Young, L. Hennighausen, and P. A. Furth. 1997. Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. *Proc. Natl. Acad. Sci. USA* 94:3425–3430.
- Lund, L. R., J. Romer, N. Thomasset, H. Solberg, C. Pyke, M. J. Bissell, K. Dano, and Z. Werb. 1996. Two distinct phases of apoptosis in mammary gland involution: Proteinase-independent and -dependent pathways. *Development* 122:181–193.
- Knight, C. H., D. Hirst, and R. J. Dewhurst. 1994. Milk accumulation and distribution in the bovine udder during the interval between milkings. *J. Dairy Res.* 61:167–177.
- Marti, A., Z. Feng, H. J. Altermatt, and R. Jaggi. 1997. Milk accumulation triggers apoptosis of mammary epithelial cells. *Eur. J. Cell Biol.* 73:158–165.
- McMahon, C. D., V. C. Farr, K. Singh, T. T. Wheeler, and S. R. Davis. 2004. Decreased expression of β 1-integrin and focal adhesion kinase in epithelial cells may initiate involution of mammary glands. *J. Cell. Physiol.* 200:318–325.
- Metcalfe, A. D., A. Gilmore, T. Klinowska, J. Oliver, A. J. Valentijn, R. Brown, A. Ross, G. MacGregor, J. A. Hickman, and C. H. Streuli. 1999. Developmental regulation of Bcl-2 family protein expression in the involuting mammary gland. *J. Cell Sci.* 112:1771–1783.
- Nicholas, K. R. 1988. Control of milk protein synthesis in the marsupial *Macropus eugenii*: A model system to study prolactin-dependent development. Pages 68–85 in *The Developing Marsupial: Models for Biomedical Research*. C. H. Tyndale-Biscoe and P. A. Janssens, ed. Springer-Verlag, Berlin, Germany.
- Nicholas, K. R., J. A. Fisher, E. Muths, J. Trott, P. A. Janssens, C. Reich, and D. C. Shaw. 2001. Secretion of whey acidic protein and cystatin is down regulated at mid-lactation in the red kangaroo (*Macropus rufus*). *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* 129:851–858.
- Nicholas, K. R., K. Simpson, M. Wilson, J. Trott, and D. Shaw. 1997. The tammar wallaby: A model to study putative autocrine-induced changes in milk composition. *J. Mammary Gland Biol. Neoplasia* 2:299–310.

- Nicholas, K. R., and C. H. Tyndale-Biscoe. 1985. Prolactin-dependent accumulation of α -lactalbumin in mammary gland explants from the pregnant tamarin wallaby (*Macropus eugenii*). *J. Endocrinol.* 106:337–342.
- Nicholas, K. R., C. J. Wilde, P. H. Bird, K. A. K. Hendry, K. Tregenza, and B. Warner. 1995. Asynchronous concurrent secretion of milk proteins in the tamarin wallaby (*Macropus eugenii*). Pages 153–170 in *Intercellular Signalling in the Mammary Gland*. C. J. Wilde, ed. Plenum Press, New York, NY.
- Nukumi, N., K. Ikeda, M. Osawa, T. Iwamori, K. Naito, and H. Tojo. 2004. Regulatory function of whey acidic protein in the proliferation of mouse mammary epithelial cells in vivo and in vitro. *Dev. Biol.* 274:31–44.
- Oftedal, O. T., D. J. Boness, and R. A. Tedmam. 1987. The behaviour, physiology, and anatomy of lactation in the Pinnipedia. *Curr. Mammal.* 1:175–245.
- Peaker, M., C. J. Wilde, and C. H. Knight. 1998. Local control of the mammary gland. *Biochem. Soc. Symp.* 63:71–79.
- Quarrie, L. H., C. V. Addey, and C. J. Wilde. 1995. Apoptosis in lactating and involuting mouse mammary tissue demonstrated by nick-end DNA labelling. *Cell Tissue Res.* 281:413–419.
- Ranganathan, S., K. J. Simpson, D. C. Shaw, and K. R. Nicholas. 1999. The whey acidic protein family: A new signature motif and three-dimensional structure by comparative modeling. *J. Mol. Graph. Model.* 17:106–113.
- Rennison, M. E., M. Kerr, C. V. Addey, S. E. Handel, M. D. Turner, C. J. Wilde, and R. D. Burgoyne. 1993. Inhibition of constitutive protein secretion from lactating mouse mammary epithelial cells by FIL (feedback inhibitor of lactation), a secreted milk protein. *J. Cell Sci.* 106:641–648.
- Schmidt, D. V., L. E. Walker, and K. E. Ebner. 1971. Lactose synthetase activity in northern fur seal milk. *Biochim. Biophys. Acta* 252:439–442.
- Sharp, J. A., K. N. Cane, C. Lefevre, J. P. Arnould, and K. R. Nicholas. 2006. Fur seal adaptations to lactation: Insights into mammary gland function. *Curr. Top. Dev. Biol.* 72:275–308.
- Simpson, K. J., and K. R. Nicholas. 2002. The comparative biology of whey proteins. *J. Mammary Gland Biol. Neoplasia* 7:313–325.
- Simpson, K. J., S. Ranganathan, J. A. Fisher, P. A. Janssens, D. C. Shaw, and K. R. Nicholas. 2000. The gene for a novel member of the whey acidic protein family encodes three four-disulfide core domains and is asynchronously expressed during lactation. *J. Biol. Chem.* 275:23074–23081.
- Skehan, P., R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J. T. Warren, H. Bokesch, S. Kenney, and M. R. Boyd. 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* 82:1107–1112.
- Strange, R., F. Li, S. Saurer, A. Burkhardt, and R. R. Friis. 1992. Apoptotic cell death and tissue remodelling during mouse mammary gland involution. *Development* 115:49–58.
- Trillmich, F., ed. 1996. *Parental Investment in Pinnipeds*. Academic Press, San Diego, CA.
- Triplett, A. A., K. Sakamoto, L. A. Matulka, L. Shen, G. H. Smith, and K. U. Wagner. 2005. Expression of the whey acidic protein (WAP) is necessary for adequate nourishment of the offspring but not functional differentiation of mammary epithelial cells. *Genesis* 43:1–11.
- Trott, J. F., K. J. Simpson, R. L. C. Moyle, C. M. Hearn, G. Shaw, K. R. Nicholas, and M. B. Renfree. 2003. Maternal regulation of milk composition, milk production, and pouch young development during lactation in the tamarin wallaby (*Macropus eugenii*). *Biol. Reprod.* 68:929–936.
- Trott, J., M. Wilson, R. Hovey, D. C. Shaw, and K. R. Nicholas. 2002. Expression of novel lipocalin-like milk protein gene is developmentally regulated during lactation in the tamarin wallaby, *Macropus eugenii*. *Gene* 283:287–297.
- Tyndale-Biscoe, C. H., and P. A. Janssens, ed. 1988. *The Developing Marsupial: Models for Biomedical Research*. Springer-Verlag, Berlin, Germany.
- Urashima, T., M. Arita, M. Yoshida, T. Nakamura, I. Arai, T. Saito, J. P. Arnould, K. M. Kovacs, and C. Lydersen. 2001. Chemical characterisation of the oligosaccharides in hooded seal (*Cystophora cristata*) and Australian fur seal (*Arctocephalus pusillus doriferus*) milk. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.* 128:307–323.
- Weaver, V. M., O. W. Petersen, F. Wang, C. A. Larabell, P. Briand, C. Damsky, and M. J. Bissell. 1997. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. *J. Cell Biol.* 137:231–245.
- Wilde, C. J., C. V. P. Addey, L. Boddy-Finch, and M. Peaker. 1995. Autocrine control of milk secretion: From concept to application. Pages 227–237 in *Intercellular Signalling in the Mammary Gland*. C. J. Wilde, ed. Plenum Press, New York, NY.