

# TRIENNIAL LACTATION SYMPOSIUM: A local affair: How the mammary gland adapts to changes in milking frequency<sup>1,2</sup>

E. H. Wall\* and T. B. McFadden†<sup>3</sup>

\*Department of Medicine, University of Vermont, Burlington, 05405; and †Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Canada T6G 2P5

**ABSTRACT:** Regular removal of milk from the mammary gland is critical to maintaining milk secretion. Early studies in rodents demonstrated that changes in milking frequency influenced mammary blood flow, as well as mammary cell number and activity. Later studies in ruminants confirmed those observations and that the response was regulated locally within the mammary gland. In addition, it was discovered that increased milking frequency (IMF) during early lactation stimulated an increase in milk production that partially persisted through late lactation, indicating long-term effects on mammary function. The local mechanisms regulating the mammary response to IMF are poorly understood, although several have been proposed. To gain insight into the mechanisms underlying the mammary response to IMF, and to identify genes associated with the response, we used a functional genomics approach and conducted experiments on dairy cows exposed to unilateral frequent milking [UFM; twice daily milking (2X) of the left udder half and 4-times daily milking (4X) of the right udder half]. Across multiple experiments, we

were unable to detect an effect of UFM on mammary cell proliferation or apoptosis. We have, however, identified distinct transcriptional signatures associated with the mammary response to milk removal and to UFM during early lactation. Sequential sampling of mammary tissue revealed that when UFM was imposed during early lactation, at least 2 sets of genes were coordinately regulated with changes in differential milk production of 4X vs. 2X udder halves. Moreover, some genes were persistently differentially expressed in 4X vs. 2X udder halves after UFM and were associated with the persistent increase in milk yield. We conclude that a coordinated transcriptional response is associated with the increase in milk yield elicited by IMF during early lactation and that the 2 sets of differentially expressed genes may be a marker for the autocrine up-regulation of milk production. Moreover, we propose that we have identified a novel form of imprinting associated with persistent alteration of mammary function, which we term “lactational imprinting.”

**Keywords:** gene expression, imprinting, lactation, mammary gland

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J. Anim. Sci. 2012.90:1695–1707  
doi:10.2527/jas2011-4790

## INTRODUCTION

In lactating animals, regular removal of milk from the mammary gland is critical to maintaining milk production. Research in both rodents and ruminants

has shown that milk secretion is diminished when the suckling stimulus is reduced, and that milk production is enhanced when the suckling frequency or intensity is increased. Therefore, the nutritional demands of the offspring partially determine or modulate the productive capacity of the gland. In livestock, an increase in demand for milk by the offspring can be mimicked by increased milking frequency (IMF;  $\geq 3$  times daily). Indeed, IMF of dairy cows is commonly used as an effective management tool to increase milk yield and production efficiency. Relative to cows milked twice daily (2X), cows milked 3 times daily (3X) generally produce 15 to 20% more milk, and milk production can be increased an additional 7% by milking 4 times daily (4X) instead of 3X (Erdman and Varner, 1995;

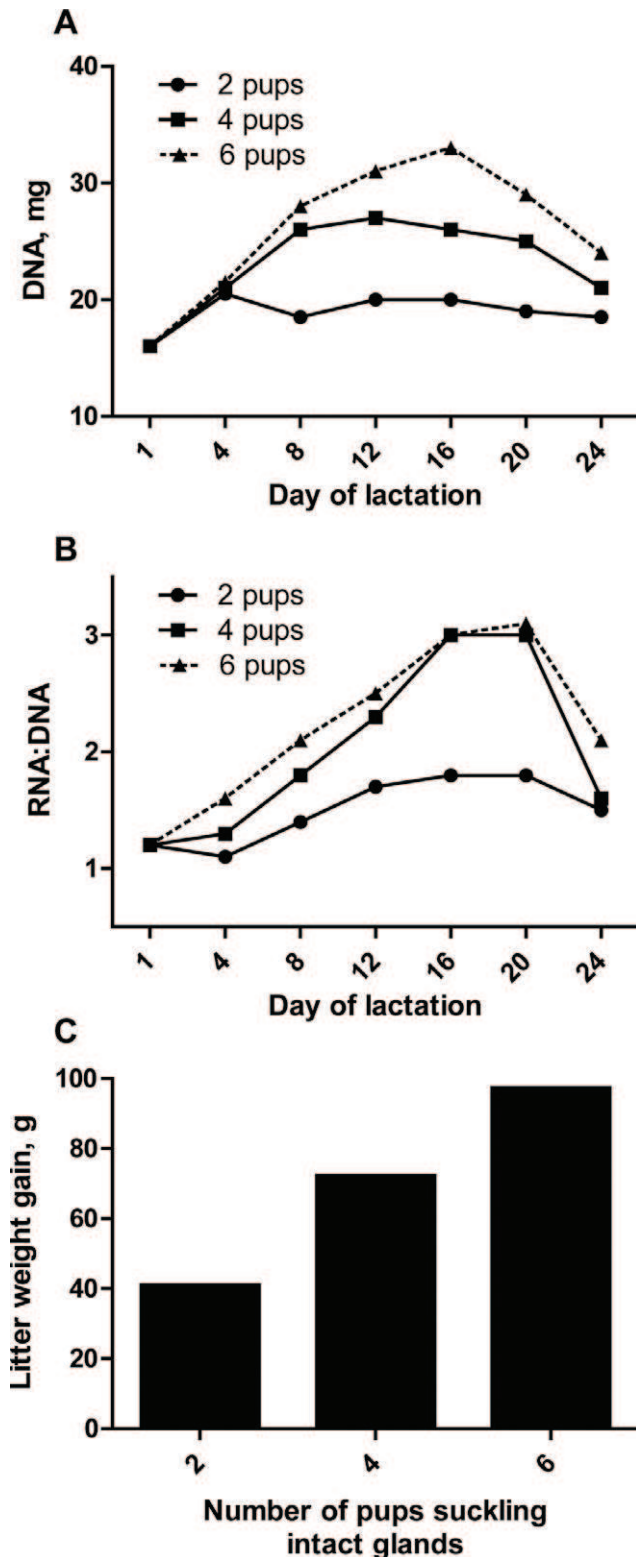
<sup>1</sup>Based on a presentation at the Triennial Lactation Symposium titled “Lactation Biology Training for the Next Generation – A Tribute to Dr. H. Allen Tucker” preceding the Joint Annual Meeting, July 10, 2011, New Orleans, LA, with publication sponsored by the American Animal Science Association and the *Journal of Animal Science*.

<sup>2</sup>This project was supported in part by National Research Initiative Competitive Grant no. 2007-35206-17983 from the USDA National Institute of Food and Agriculture, the Alberta Livestock and Meat Agency, and the Natural Sciences and Engineering Research Council of Canada.

<sup>3</sup>Corresponding author: thomas.mcfadden@ualberta.ca

Received August 8, 2011.

Accepted October 16, 2011.



**Figure 1.** Litter size is positively correlated with mammary cell number (A), activity (B), and milk yield (C) in lactating rats (redrawn from Tucker, 1966). On d 3 of lactation, litters were adjusted to 2, 4, or 6 pups per 6 glands (thoracic teats were ligated). Throughout lactation, mammary glands were collected and nucleic acid content measured on the days indicated. The amount of DNA was used as an indicator of mammary cell number, whereas the RNA:DNA ratio was used as an indicator of mammary cell activity. Milk yield was estimated by litter weight gain, which was measured on d 16 of lactation.

Stelwagen, 2001; Stockdale, 2006). In addition, the stimulus of IMF for a short period (i.e., 2 to 3 wk) during early lactation is sufficient to increase milk production through late lactation, long after 2X is resumed (Wall and McFadden, 2008). This indicates that the mammary gland is especially sensitive during early lactation to the demands of the offspring, which influence the shape of the lactation curve.

The mechanisms underlying the response of the mammary gland to milk removal are not well understood; however, experiments in both rodents and ruminants have shown that changes in the frequency of milk removal can influence mammary cell number and activity (Hadsell et al., 2007; Wall and McFadden, 2008). In addition, the increase in milk yield associated with IMF is regulated locally within the mammary gland (Wall and McFadden, 2007a). The objectives of this review were to summarize the literature on the effect of milking frequency or suckling intensity on mammary function in rodents and ruminants, and to discuss the physiological bases and potential mechanisms involved in the response to IMF and the long-term alteration of mammary function.

### THE EFFECT OF SUCKLING INTENSITY ON MAMMARY DEVELOPMENT AND MILK PRODUCTION IN RODENTS

The number of pups suckling the dam has a marked effect on mammary function in lactating rodents. Specifically, increased suckling frequency was associated with an increase in milk production, as measured by litter weight gain (Thatcher and Tucker, 1968; Russell, 1980). Russell (1980) reported that relative to rats suckling 1 pup, rats suckling 10 pups produced 10 times more milk during early lactation, and the difference increased to 20 times more milk by the middle of lactation. The increase in milk yield seen with greater numbers of suckling pups was also associated with an increase in mammary cell number and activity, indicated by DNA content and the RNA:DNA ratio, respectively (Figure 1; Tucker, 1966). The increase in DNA and RNA was observed within 24 h of increased suckling intensity, indicating rapid regulation in response to increased demand of the offspring (Tucker, 1966; Tucker et al. 1967a). In addition, because litter weight gain was highly correlated with the RNA:DNA ratio, it is thought that mammary cell activity is the driving force for milk yield in rodents (Tucker, 1966; Tucker et al., 1967a).

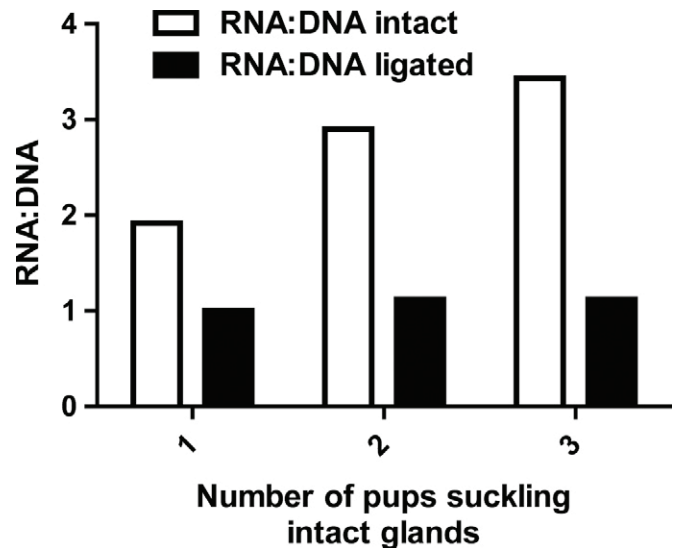
### HORMONAL RESPONSE TO CHANGES IN SUCKLING INTENSITY IN RODENTS

Because both prolactin (PRL) and oxytocin (OT) are released during suckling, and PRL stimulates mam-

mary cell differentiation, it was hypothesized that the mammary response to increased suckling intensity was hormonally regulated. Indeed, Tucker et al. (1967a,b) observed a linear increase in pituitary gland content of PRL with increasing litter size. However, because mammary cell RNA was 21-fold greater in rats suckled ad libitum relative to those suckled once daily, but pituitary PRL content was only 2-fold greater, it was concluded that changes in PRL secretion were only partially responsible for the increase in mammary cell activity and consequent milk production. Administration of OT to rats during extended lactation was associated with preservation of mammary function, but not with a significant increase in milk yield (Thatcher and Tucker, 1970). In that study, treatment of lactating rats with cortisol elicited a 2-fold increase in litter weight gain; therefore, the authors concluded that cortisol was limiting to milk production during late lactation in rodents (Thatcher and Tucker, 1970). However, because no hormone treatments elicited as marked an effect on milk production as seen with increased suckling intensity, it is likely that local factors within the mammary gland regulate the response, or mediate the response, of the mammary gland to systemic hormones. Indeed, local regulation of the response of the mammary gland to OT has been observed. In lactating rats, milk stasis was associated with an increase in intramammary pressure and lactose content of the mammary gland in response to exogenous OT (Kuhn et al., 1973).

### LOCAL REGULATION OF MAMMARY FUNCTION IN RODENTS

Local regulation of mammary cell number and secretory activity has been observed in lactating rats. In teat ligation experiments, selected teats were ligated, and pups were allowed to continue suckling intact glands. Tucker and Reece (1963) and Tucker (1966) observed that after 24 h of milk stasis, the RNA:DNA ratio in ligated glands had decreased by 31%, which they interpreted as a decrease in mammary cell activity (Figure 2). The authors suggested that during milk stasis, intact (suckled) glands were able to take up more nutrients and hormones from the circulation than the sealed glands and that this may explain the observed increase in milk yield, mammary cell number, and mammary cell secretory activity (Tucker, 1966; Tucker et al., 1967a). Increased nutrient availability to suckled glands could be mediated by local changes in blood flow. Silver (1956) reported that within 100 h of sealing select teats and subsequent engorgement of glands with milk, mammary involution had taken place, and capillaries were empty and collapsed. When pups were allowed to resume suckling of the previously sealed teats, the capil-



**Figure 2.** Local regulation of mammary cell activity in lactating rats (redrawn from Tucker, 1966). On d 3 of lactation, litters were adjusted to 1, 2, or 3 pups per 3 mammary glands (all 6 thoracic teats, plus all 3 left or right abdominal-inguinal teats were ligated, leaving 3 intact glands). Mammary glands were collected on d 16 of lactation, and nucleic acid content was measured. Mammary cell DNA content was used to estimate changes in mammary cell number, which showed a similar pattern.

lary bed was promptly refilled with blood and mammary function was restored (Silver, 1956). This occurred even when the contralateral glands were suckled, indicating that mammary blood flow was indeed under the control of local factors and not systemic hormones.

Taken together, these observations support the concept that removal of milk from the mammary gland of rodents stimulates mammary cell number and activity, blood flow, nutrient availability, and consequent milk yield. In addition, although systemic hormones appear to play a role, the response is clearly regulated locally by factors within the mammary gland. The mechanistic basis for these events, however, is still poorly understood.

### EFFECT OF INCREASED MILKING FREQUENCY ON MAMMARY DEVELOPMENT AND MILK PRODUCTION IN RUMINANTS

Consistent with observations in rodents, IMF, whether by machine-milking or by a suckling calf, elicits an increase in milk production in ruminants (Everitt and Phillips, 1971; Erdman and Varner, 1995; Stelwagen, 2001; Stockdale, 2006). This observation was made as early as the late 1800s, when it was reported that there was a positive relationship between milking frequency and milk production, and that producers should consider milking 3X (Hills, 1890). Since then, the consistent response to IMF has led to the adoption of 3X on many dairy farms. Although more recent work reported a fixed incremental increase in milk yield of 3.5 kg/d upon

changing from 2X to 3X milking (Erdman and Varner, 1995), modern-day adjustment factors used by DHIA to compare milk production of cows milked 2X with those milked 3X range from 12 to 14%, depending on the parity of the cow (VanRaden et al., 1999). Therefore, the practice of 3X has proven to be an effective management strategy to increase milk production.

In addition to the stimulatory effect of 3X on milk production, IMF (i.e., 4X to 6X) for the first few weeks during early lactation elicits an increase in milk production that partially carries over through the remainder of that lactation, even after the milking frequency is returned to 2X or 3X (Bar-Peled et al., 1995; Hale et al., 2003; Dahl et al., 2004b). Subsequently, numerous experiments confirmed that IMF during early lactation was associated with both acute and persistent increases in milk production (Wall and McFadden, 2008). Relative to cows milked 2X, those milked 4X during the first 3 wk of lactation followed by 2X thereafter produced 8.8 kg/d more milk during 4X, and 2.6 kg/d more milk for the remainder of lactation (Hale et al., 2003). Similar responses have been observed in field studies (Dahl et al., 2004b; Soberon et al., 2011). For dairy producers, these findings represent an exciting management opportunity: that an initial investment in labor can increase milk production efficiency for the remainder of lactation. From a mechanistic standpoint, these observations present some interesting questions. First, why is the mammary gland especially responsive in early lactation to the demands of the offspring? Second, it makes biological sense that the needs of the neonate influence productivity of the mammary gland, but why does an increase in milk production persist, long after cessation of IMF? Finally, what are the underlying mechanisms for each of these effects?

### **CELLULAR RESPONSE TO INCREASED MILKING FREQUENCY**

Many researchers have hypothesized that IMF stimulates milk yield via an increase in mammary cell number or activity or both (Bar-Peled et al., 1995; Stelwagen and Knight, 1997; Sanders et al., 2000; Hale et al., 2003), both of which may be critical to improved lactation performance (Capuco et al., 2003). Hillerton et al. (1990) observed an increase in activity of mammary enzymes, protein and lactose synthesis, DNA synthesis, and alveolar area in response to IMF and concluded that cellular differentiation and proliferation were enhanced by IMF. Hale et al. (2003) reported an increase in mammary cell proliferation on d 7 of lactation in cows that were milked 4X on d 1 to 7 of lactation compared with cows milked 2X; however, cows milked 4X on d 4 to 7 did not differ from 2X cows. Norgaard et al. (2005) reported that de-

spite an 18% increase in milk yield of dairy cows milked 4X during d 119 to 126 of lactation, there was no effect of IMF on cell death, proliferation, or enzyme activities in the mammary gland. In agreement with those observations, we observed that relative to 2X, 4X did not affect mammary epithelial cell proliferation on d 7 of lactation (Wall et al., 2006). Therefore, in contrast to rodents, an effect of IMF on mammary cell number in dairy cows has not been consistently observed. Discrepancies in reported effects of IMF on mammary cell proliferation may be due to limitations in assay sensitivity or normal animal variation across experiments or both. In addition, it is possible that, as seen in rodents, the increase in milk yield is mediated by changes in mammary cell activity or mammary blood flow or both.

### **EFFECT OF MILKING FREQUENCY ON MAMMARY BLOOD FLOW**

Mao and Caruolo (1973) reported that mammary blood flow was inversely related to the amount of milk accumulated in the gland and that decreased milk secretion during milk stasis may be mediated by a decrease in availability of nutrients to the mammary gland. Similarly, during extended milk stasis in lactating goats, blood flow to the mammary gland decreased linearly over 36 h (Stelwagen et al., 1994). Stelwagen et al. (1994) suggested that during milk stasis, the decline in mammary blood flow may be the result of negative feedback from the gland due to a reduction in demand for milk precursors. Farr et al. (2000) reported that extended milk stasis in lactating goats resulted in a 50 to 75% decrease in mammary blood flow and capillary permeability, as well as a marked regression of the vasculature, in agreement with previous observations in mice (Silver, 1956). The results of this research support the concept that during milk stasis, blood flow to, and metabolic capacity of, the mammary gland is impaired (Farr et al., 2000). In contrast to the negative effect of milk stasis on mammary blood flow, a positive relationship has been observed between mammary blood flow and IMF. During hourly milking (Farr et al., 2000) or IMF (Bequette and Douglass, 2010) of lactating goats, blood flow to the mammary gland was acutely increased. In addition, milk yield of lactating goats increased within 2 h of an experimental increase in mammary blood flow via vasodilatation (Prosser et al., 1990). After the treatments stopped, however, milk yield decreased to pretreatment quantities. Despite these observations, IMF does not always stimulate an increase in mammary blood flow (Maltz et al., 1984), and an increase in mammary blood flow does not always elicit an increase in milk yield (Prosser et al., 1994; Lacasse and Prosser, 2003). Therefore, although

mammary blood flow and milk yield are closely associated, they are not always causally linked, and there appear to be other limiting factors involved.

### HORMONAL RESPONSE TO INCREASED MILKING FREQUENCY

As mentioned previously, OT and PRL, among other hormones, are released during suckling and milking (Tucker et al., 1975; Carruthers and Hafs, 1980; Akers and Lefcourt, 1982), and it has long been hypothesized that they are involved in regulating the galactopoietic effects of IMF on milk production. Along with increased milk production, Bar-Peled et al. (1995) observed greater concentrations of OT and PRL in circulation of cows that were frequently milked or suckled. Because PRL is involved in differentiation of the mammary gland, and the magnitude of milking-induced PRL release declines concomitantly with the decrease in milk production as lactation progresses (Koprowski and Tucker, 1973), PRL has also been suggested as a candidate regulator of the effects of IMF on milk production (Dahl et al., 2004a). Four times daily milking or 2X plus PRL injections increased milk production relative to 1X (Crawford et al., 2004). However, the effects of PRL injection on mammary cell growth and gene expression differed from the effects of IMF, indicating that those treatments increased milk production via separate mechanisms (Wall et al., 2006). In addition, treatment of early- and midlactation dairy cows with exogenous PRL had no effect on milk yield (Plaut et al., 1987). Therefore, it appears that IMF stimulates milk production via local factors, whereas PRL treatment may also involve systemic pathway(s).

Because OT is responsible for milk ejection, and the release of OT is elicited by the presence of the calf, cows allowed to suckle their calf in addition to machine milkings are thought to have more efficient milk ejection than cows that are machine milked only (Everitt and Phillips, 1971; Krohn, 2001). In addition, treatment with exogenous OT was associated with increased milk production of dairy cows (Nostrand et al., 1991; Ballou et al., 1993; Lollivier and Marnet, 2005). In the absence of milk removal, however, exogenous OT had no effect on milk yield (Linnerud et al., 1966). Therefore, it is possible that OT is involved in regulating the increase in milk production elicited by IMF or suckling, perhaps by allowing for more complete milk removal and a decrease in negative feedback on the gland by factors in milk or intramammary pressure. Clearly, as shown in rodents, removal of milk from the mammary gland is critical for eliciting changes in local factors that are limiting to milk production. In addition, local regulatory mechanisms influence the response of the mammary gland to hormones in the circulation.

### LOCAL REGULATION OF MILK

## PRODUCTION AND MAMMARY FUNCTION

As discussed above, there is substantial evidence that milk production is regulated by local factors, within the mammary gland, as well as systemic factors (Wilde et al., 1995). Early studies involving application of different milking frequencies to udder halves provided strong evidence for local regulation of milk production, and increases in milk yield from 8.4 to 32% in the more frequently-milked udder half were observed (Ludwick et al., 1941; Cash and Yapp, 1950; Agarwala and Sundaresan, 1955; Claesson et al., 1959). Morag (1973) reported that milk production of the frequently milked udder half increased within 24 h, and the incremental response was independent of previous milk production. By contrast, once daily milking (1X) is associated with a marked reduction in milk yield, relative to 2X (Stelwagen and Knight, 1997), and this response sometimes persisted even after cessation of treatment (Bernier-Dodier et al., 2010). Although reduced milking frequency (e.g., 1X or outright cessation of milking) has often been used to study the local factors involved in the regulation of milk production, it appears that IMF acts on the gland via distinct mechanisms (i.e., not simply opposite responses of the same mechanisms). Whereas 1X and milk stasis elicit drastic changes in milk yield and mammary remodeling, the response of the gland to IMF may be mediated by changes in mammary cell activity.

In addition to the effect of milk removal on mammary blood flow and uptake of nutrients for milk synthesis, the mammary response to milk removal may be regulated by changes in intramammary pressure or by chemical factors present in the milk or milk fat.

#### *Intramammary Pressure*

Because accumulation of milk causes intramammary pressure to increase, it is not surprising that pressure has been investigated as a potential regulator of mammary blood flow and milk secretion. Infusion of air or milk into the mammary glands of goats was associated with an increase in intramammary pressure and a linear decrease in mammary blood flow (Pearl et al., 1973). The infusion of only 1 udder half revealed that this response is regulated locally within the gland, because blood flow of adjacent glands was unaffected (Pearl et al., 1973). Peaker (1980) reported that loss of mammary cell secretory activity during milk stasis of lactating goats was caused by an increase in intramammary pressure, and not by a decrease in mammary blood flow. An increase in intramammary pressure, however, did not always result in a decrease in milk production (Henderson and

Peaker, 1984). Therefore, the relationship between intramammary pressure, mammary blood flow, and milk removal remains unclear. It is possible that intramammary pressure may indeed be a local mediator of mammary function, but its role may change with physiological state, metabolic status, and stage of lactation.

### ***Feedback Inhibitor of Lactation***

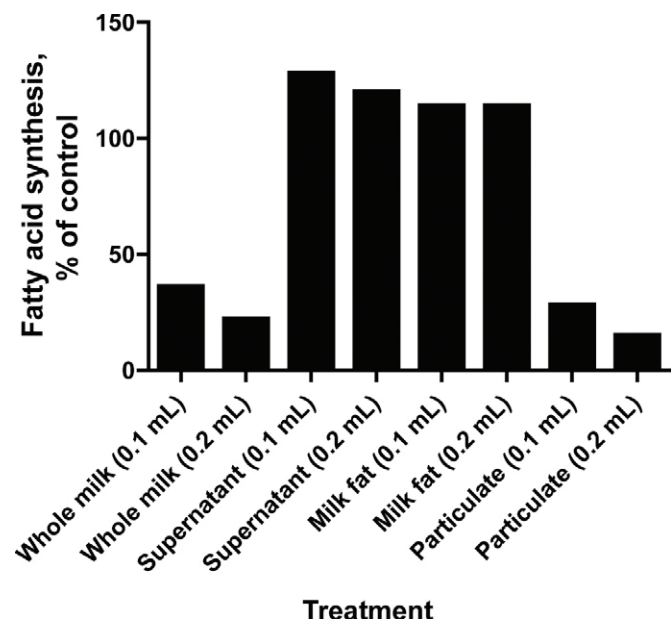
Linzell and Peaker (1971) hypothesized that a chemical in milk negatively regulates milk secretion in the absence of milk removal. Subsequently, a small glycoprotein in milk was reported to reversibly inhibit casein and lactose synthesis in a dose-dependent manner (Wilde et al., 1987). This glycoprotein was named feedback inhibitor of lactation (**FIL**). It was reportedly both synthesized and secreted by mammary epithelial cells and was a component of the whey fraction of milk. It was proposed that FIL is the major autocrine regulator of milk secretion and functions to adjust milk production to meet, but not exceed, the nutritional demands of the offspring (Peaker and Wilde, 1987). The mechanisms underlying this regulation have not been fully explained; however, Peaker and Wilde (1987) originally proposed that the mammary gland responds to removal of FIL in a sequential manner consisting of an immediate response that increases milk secretion within hours of milk removal, an acute response that increases mammary cell differentiation after several days of IMF, and finally a long-term response that increases mammary cell proliferation after several weeks or months of IMF. Later studies provided some evidence that FIL inhibits milk production by interfering with the casein secretory pathway (Rennison et al., 1993; Burgoyne and Wilde, 1994). Unfortunately, no further reports have been published on the mechanism by which FIL may regulate milk secretion. To the contrary, research on this protein has apparently not been pursued since the 1990s, and the identity of the putative protein and its role in the mammary gland have yet to be confirmed.

### ***Negative Feedback on Milk Fat Synthesis***

Before reports on FIL, it was observed that mammary synthesis of fatty acids was regulated by a factor within the milk fat itself (Levy, 1963, 1964). This research, however, received much less attention than the FIL literature. Levy (1964) observed an accumulation of fat within 12 h of weaning and a consequent diminution of fatty acid synthesis in the mammary gland of lactating rats. By 24 h, fatty acid synthesis was reduced by 90%, and lactose was reabsorbed into the bloodstream. The synthesis of fatty acids was restored, however, when pups were returned to the mother to suckle (Levy, 1964).

Teat-ligation experiments showed that the regulation occurred at the level of the individual mammary gland, because intact (suckled) glands continued to synthesize milk and milk fat (Levy, 1964). Further studies showed that addition of whole milk to culture medium markedly inhibited the synthesis of fatty acids by rat mammary tissue explants in a dose-dependent response. Subsequent analysis revealed that the inhibitory activity was acting on acetyl CoA carboxylase and was not associated with milk fat itself, but with the particulate fraction of milk (Figure 3; Levy, 1964). Levy (1964) speculated that the inhibitor was bound to microsomes in the milk.

More recently, inhibition of mammary lipogenesis by medium chain fatty acids has been reported (Agius and Williamson, 1980; Heesom et al., 1992). Heesom et al. (1992) suggested that FIL may regulate lactose and casein synthesis, whereas fat synthesis may be regulated by a negative feedback mechanism involving medium-chain fatty acids. To test this hypothesis, Peaker and Taylor (1994) investigated the effect of milk fat on litter weight gain in mice. Intraperitoneal injection of whole milk, which contains milk fat globules, into lactating mice inhibited litter growth, whereas skim milk, which was supposed to contain FIL, or fractions of milk fat globules alone had no effect. The authors concluded that there is no negative feedback mechanism associated with milk fat. This conclusion, however, seemed particularly dismissive because it did not account for the inhibitory effect of whole milk. In addition, their results did not prompt them to question a role for FIL, which had no apparent effect on litter weight gain. Perhaps, coincidentally, that report was one of the last published



**Figure 3.** Inhibition of fatty acid synthesis by milk fractions. Mammary glands from lactating rats were incubated with various milk fractions, and fatty acid synthesis was assayed as described by Levy (1964).

primary research articles investigating a role for FIL in the mammary gland.

Certainly, there is evidence for the existence of at least 2 types of chemical negative feedback mechanisms involved in the regulation of milk synthesis and secretion. Moreover, it is probable that there are other feedback mechanisms that have yet to be discovered. Indeed, serotonin (Hernandez et al., 2008) and a peptide fragment of  $\beta$ -casein (Shamay et al., 2002; Silanikove et al., 2009) have been recently proposed as feedback regulators of milk production. Such factors may act on synthesis or secretion of particular components of milk or both, or they may have general effects. It makes biological sense that a metabolically expensive process such as lactation would be tightly regulated by a variety of local mechanisms to prevent overproduction in the absence of milk removal.

Interestingly, fur seals do not undergo inhibition of milk secretion or mammary involution during prolonged absence of milk removal (reviewed by Sharp et al., 2006). During lactation, these animals go through cycles of suckling their young on land and foraging for food at sea for up to 30 d at a time. During foraging, milk secretion continues and mammary function is maintained so that the seals can suckle their young when they return to shore. It has been suggested that fur seal lactation has evolved to override the influence of local negative feedback mechanisms to accommodate their foraging cycles and continue to rear their offspring successfully (Sharp et al., 2006). Moreover, this adaptation is thought to be regulated at the transcriptional level (Sharp et al., 2008). This is an exciting and active area of study. Once the mechanisms of local regulation and negative feedback are understood, and the genes involved are identified, there may be an opportunity to identify limits on milk secretion and improve milk production efficiency of dairy animals.

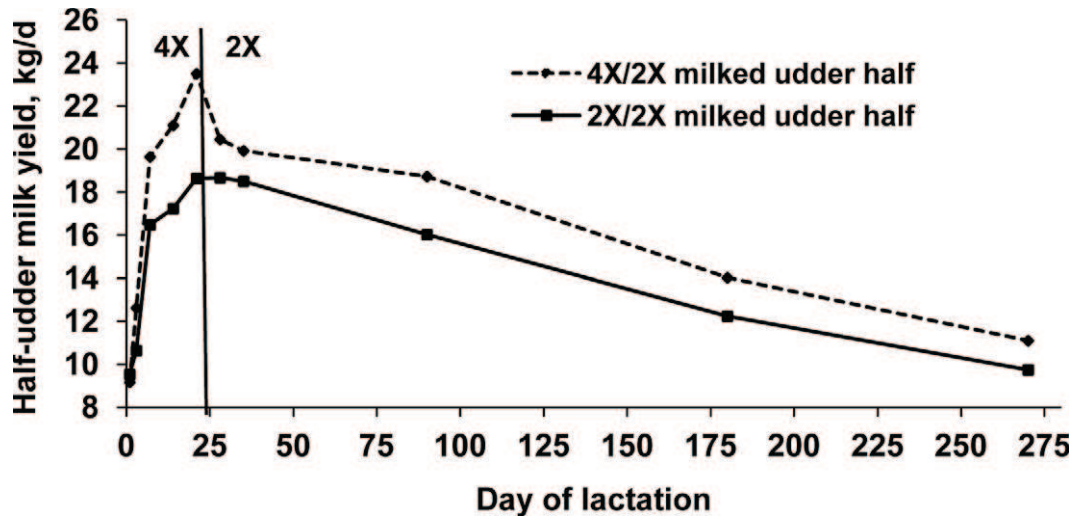
### **UNILATERAL FREQUENT MILKING: A POWERFUL APPROACH TO MECHANISTIC QUESTIONS**

Based on published reports that IMF in early lactation elicited a persistent increase in milk production, and that milk yield is regulated locally within the gland, we adopted a unilateral frequent milking (UFM) model to address some mechanistic questions about the milk yield response and associated changes in mammary development and function. The half-udder design is a statistically powerful model because it eliminates variation between animals due to environmental factors, nutrition, and genetics. Both udder halves are theoretically exposed to the same systemic factors; hence, it is possible to isolate responses to different milking frequencies to local

regulation at the level of the mammary gland. We have used this model and a functional genomics approach to 1) determine whether the acute and persistent milk yield responses are regulated locally within the gland vs. systemically by hormones; 2) investigate effects of timing and duration of IMF on milk yield responses; and 3) determine the effects of IMF in early lactation on mammary cell proliferation, apoptosis, and gene expression.

### ***Milk Yield Response to Increased Milking Frequency in Early Lactation is Locally Regulated***

Although both acute and persistent milk yield responses to IMF in early lactation have been consistently observed, it was not known whether these responses were regulated by hormones, by local factors within the mammary gland, or by the combination of the two. To investigate this question, we assigned cows to UFM (4X of the right udder half and 2X of the left udder half) on d 1 to 21 of lactation, followed by 2X for the remainder of lactation (Wall and McFadden, 2007a). Lactation curves of 2X and 4X-2X udder halves are presented in Figure 4. We observed a rapid and marked increase in milk yield of the 4X udder halves during UFM that peaked on d 21 of lactation. After cessation of UFM, milk yield of 4X udder halves initially decreased but remained greater than that of 2X udder halves through d 270 of lactation (Figure 4; Wall and McFadden, 2007a). Moreover, when the half-udder milk yields were projected to the equivalent of a whole udder basis (Table 1), the acute and long-term milk yield responses to IMF were consistent with those reported by Bar-Peled et al. (1995) and Hale et al. (2003). Therefore, our results indicate that both the acute and persistent effects of IMF during early lactation are regulated by local factors within the mammary gland. Interestingly, the increase in milk yield during IMF treatment was similar across experiments (Table 1), even though different milking intervals were used. Whereas Bar-Peled et al. (1995) used evenly spaced 4-h milking intervals, we (Wall and McFadden, 2007a) and Hale et al. (2003) used uneven milking intervals. For example, in Wall and McFadden (2007a), milking intervals of 3 and 9 h were used. Cows were milked at 0230 and 1430 h, and the 2 extra milkings, during which only the right udder half was milked, took place at 0530 and 1730 h. In a preliminary study, we found that an interval as short as 1-h is sufficient to elicit both an acute and a persistent increase in milk production of 4X udder halves (Kissell et al., 2007). Therefore, even on small dairy farms with short milking sessions, IMF in early lactation can be used to enhance lactation performance. In general, the finding that the milk yield response is not entirely dependent on milking interval makes it unlikely that either intramammary pressure or the volume of milk



**Figure 4.** Acute and persistent increases in milk yield in response to unilateral 4-times daily milking of dairy cows on d 1 to 21 of lactation (Wall and McFadden, 2008).

removed are the main factors regulating the response. Rather, it appears that it is the stimulus of remilking that causes increased milk production both during and after IMF treatment. Our observation that removal of residual milk, which has increased milk fat, is sufficient to elicit an increase in milk yield is consistent with previous speculation that a component of milk fat is involved in local regulation of milk production (Levy, 1963, 1964). In addition, although it appears to be mainly a local effect, it is possible that there is an interaction between milk removal and the hormones released at milking, and the combination of these factors elicits a stimulatory effect on milk yield.

#### ***4X-Milking for Only 2 wk can Elicit a Persistent Increase in Milk Yield***

As mentioned previously, there appears to be a “window” of time wherein the mammary gland is especially responsive to IMF. The duration of IMF that is required to elicit a carryover effect on milk yield has been progressively reduced from the first 10 wk (Moss and O’Grady, 1978; Thomas et al., 1978), to the first 6 wk (Bar-Peled et al., 1995; Sanders et al., 2000), to the first 3 wk of lactation (Hale et al., 2003; Dahl et al., 2004b; Wall and McFadden, 2007a). It was not known how a still shorter duration or altered timing of IMF during early lactation would affect the persistent milk yield response; however, because added labor costs associated with extra milkings accrue only during IMF, it was of great interest to determine the minimal duration of IMF needed to elicit a carryover effect on milk yield. In addition, if the duration, rather than the timing, of IMF was critical to the milk yield response, it would indicate that the underlying mechanisms are triggered only after surpassing a threshold of exposure to stimulus, and only then would elicit persistent effects on

milk yield. To answer this question, we assigned cows to UFM on d 1 to 14 or d 7 to 21 of lactation (Wall and McFadden, 2007b). We observed an acute milk yield response in both treatments; and a significant carryover effect in the d 7 to 21 group. There was also a carryover effect for the d 1 to 14 group at some time points, but overall it was not significant. Our results demonstrate that within the first 21 d of lactation, an interval of IMF as short as 2 wk can elicit a persistent increase in milk production. However, the carryover response was smaller (nonsignificantly) than that obtained after UFM for 21 d. Further narrowing of this “window” within the first 21 d of lactation, as well as characterization of the cellular response, could provide insight into the mechanisms underlying the receptiveness of the mammary gland to stimulus during this time.

#### ***Unilateral Frequent Milking Does Not Affect Mammary-Cell Population Dynamics***

A recurring objective of our experiments has been to determine the effects of IMF on mammary cell proliferation and apoptosis. This is logical, because a net increase in the population of mammary secretory cells could explain the persistent increase in milk production (Capuco et al., 2003). Moreover, previously reported effects of IMF on mammary cell proliferation have been inconsistent. Using our UFM model, we biopsied mammary tissue at several times during and after UFM and found no difference between 2X and 4X glands in rates of mammary epithelial cell proliferation or apoptosis (Wall et al., 2008; Wall and McFadden, 2010). We then hypothesized that the milk yield response to IMF in early lactation is mediated by changes in mammary cell activity, and we conducted experiments to investigate changes in mammary gene expression to gain



**Table 1.** Validation of a unilateral frequent milking model (UFM; twice daily milking, 2X, of the left udder half and 4 times daily milking, 4X, of the left udder half for d 1 to 21 of lactation; Wall and McFadden, 2007a)<sup>1</sup>

Reference	Treatment	Treatment duration, d	Increase in milk yield during treatment, kg/d	Increase in milk yield after treatment, kg/d <sup>2</sup>
Bar-Peled et al., 1995	6X vs. 3X	1 to 42	+ 7.3	+ 5.1
Hale et al., 2003	4X vs. 2X	1 to 21	+ 8.6	+ 2.6
Wall and McFadden, 2007a	4X vs. 2X	1 to 21	+ 7.1	+ 3.6

<sup>1</sup>Observed milk yield response to UFM was adjusted to a whole udder basis and compared to the results of Bar-Peled et al., 1995 and Hale et al., 2003, who applied different milking frequencies to cows (whole udders).

<sup>2</sup>Milk yield was recorded through d 126 (Bar-Peled et al., 1995), 300 (Hale et al., 2003), or 270 of lactation (Wall and McFadden, 2007a).

insight into the underlying mechanisms and cellular functions involved.

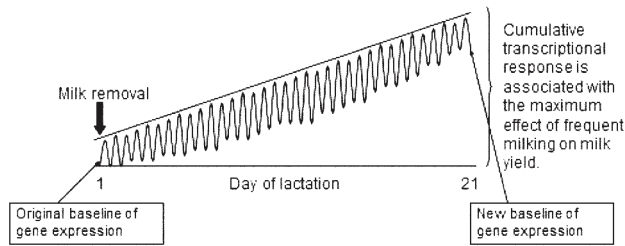
### ***Effects of Unilateral Frequent Milking on Mammary Gene Expression***

To gain insight into the mechanisms and pathways potentially involved in the milk yield response to IMF, we conducted microarray experiments to compare the transcriptomes of 4X and 2X udder halves. The first experiment was designed to identify genes differentially expressed in association with the rapid and marked increase in milk yield during UFM. A closely related objective was to quantify the acute transcriptional response of the mammary gland to milk removal, *per se*, to distinguish it from responses that might be unique to UFM. We had previously shown that expression of some genes involved in the IGF axis were regulated both by milk removal and IMF (Wall and McFadden, 2010). Cows were assigned to UFM on d 1 to 21 of lactation, and mammary biopsies were obtained from both udder halves on d 5, either immediately after milking only the 4X udder half or 2.5 h after milking both udder halves (Wall and McFadden, 2010; Wall et al., 2012). We then used microarray analysis to identify genes that were differentially expressed in each response. The results of that study revealed that on d 5 of lactation, expression of 855 genes was acutely regulated by milk removal, but no genes were uniquely associated with the sustained effect of UFM (Wall et al., 2012). We concluded that a subset of the genes that respond acutely to milking must also regulate the increase in milk yield during IMF.

To illustrate the results of these experiments, we present a hypothetical model that integrates the transcriptional responses of the mammary gland to acute milk removal and to 4X milking (Figure 5). Inspired by Fluck (2006), who proposed a very similar model to depict the acute transcriptional response of skeletal muscle to repeated bouts of exercise, it also fits our observations on the mammary gland. It accommodates our observation that the expression of 855 genes was acutely regulated by milk removal, and a discrete subset of those also responded to 4X, but none were uniquely regulated by 4X milking (Wall et al., 2012). In the model, each milk-

ing is associated with an acute transcriptional response, which is represented by the peaks in the diagram. In 2X udder halves, the response would occur twice daily, whereas in 4X udder halves, the response occurs 4 times daily. Early in the response to IMF (e.g., on d 5), gene expression returns to baseline between milkings. We propose that the continued stimulus of 4X milking for 21 d elicits a new, increased baseline of gene expression. This “adaptive” transcriptional response may be associated with the maximum difference in milk yield between 2X and 4X udder halves (Figure 4).

Having identified genes associated with both the acute response of the mammary gland to milk removal and with the early response to 4X milking, we sought to determine whether their expression was also regulated during later stages of the response to IMF. Specifically, we hypothesized that differential expression of those genes was causally related to regulation of milk yield. Therefore, predictable differences in gene expression should accompany the characteristic milk yield response depicted in Figure 4. In particular, differential expression of candidate genes should be evident at the peak response on d 21, during the rapid decrease in milk yield of 4X udder halves after cessation of UFM, and during the carryover milk yield response thereafter. Cows were assigned to UFM on d 1 to 21 of lactation, and mammary biopsies were obtained on d 21, 23, and 40 of lactation, 2.5 h after both udder halves were milked. The results of this experiment revealed that the differential expression (4X vs. 2X) of 75 genes changed significantly over time (Wall et al., 2008). Those genes segregated into 2 clusters based on the temporal pattern of differential expression. Differential expression of genes in Cluster 1 was negatively associated with differential milk yield from d 5 to 23 of lactation ( $r = -0.94$ ; E. H. Wall and T. B. McFadden, unpublished data). That is, expression of those genes was down regulated in 4X udder halves on d 21 when the difference in milk yield most favored the 4X halves. By contrast, differential expression of genes in Cluster 2 was positively associated with differential milk yield from d 5 to 23 of lactation ( $r = 0.75$ ; E. H. Wall and T. B. McFadden, unpublished data). Many of these genes were among those previously identified as responsive to IMF of dairy cows (Connor et al., 2008).



**Figure 5.** Schematic illustration of the hypothetical acute response of the mammary gland to milk removal and the long-term response to frequent milking. Each peak represents the transcriptional response to each milk removal event. Acute transcriptional responses return to baseline between milkings. The repeated stimulus of frequent milking over 21 d of lactation may establish a new baseline of gene expression. Concept inspired by Fluck (2006).

In addition, we concluded from our previous experiment (Wall et al., 2012) that some of these genes were acutely regulated by removal of milk from the mammary gland and must mediate the milk yield response to 4X milking. The results of the sequential biopsy experiment support that conclusion and confirmed genes that we had previously identified as putative responders to 4X milking (Wall and McFadden, 2010; Wall et al., 2012). Moreover, 30 of the genes in Cluster 1, but none in Cluster 2, remained differentially expressed on d 40, indicating that they may regulate the carryover effect on milk yield, which remains increased long after cessation of IMF (Wall et al., 2008). This, combined with the coregulation of these genes over time, further implicates them as members of a common pathway involved in the autocrine regulation of milk production.

Based on the coordinated changes in gene expression, we propose that we have identified a transcriptional “signature” associated with changes in mammary function and milk production in response to IMF. Coordinated transcriptional responses to stimuli have been previously described in other tissues. For example, in response to an increase in physiological demand (i.e., exercise), skeletal muscle undergoes a process of coordinated changes at the transcript level that coincide with enhanced muscular function (Fluck, 2006; Hoppeler et al., 2007). Similar observations have been made in nervous tissue, and the effect is referred to as “synaptic plasticity” (Levenson and Sweatt, 2006). Coregulation of genes may indicate an adaptive response to a physiological challenge, and it has been suggested that such transcriptional changes represent a strategy to maximize tissue function in response to increased demand (Fluck, 2006). Our data indicate a similar process may operate in the mammary gland, such that an increase in demand (i.e., IMF) during early lactation improves mammary function (i.e., increased milk production), and the response is mediated by coordinated changes in expression of key genes. Three crucial questions remain to be answered. First, what is the functional response underlying the increase in milk production? Second, how do the genes we have iden-

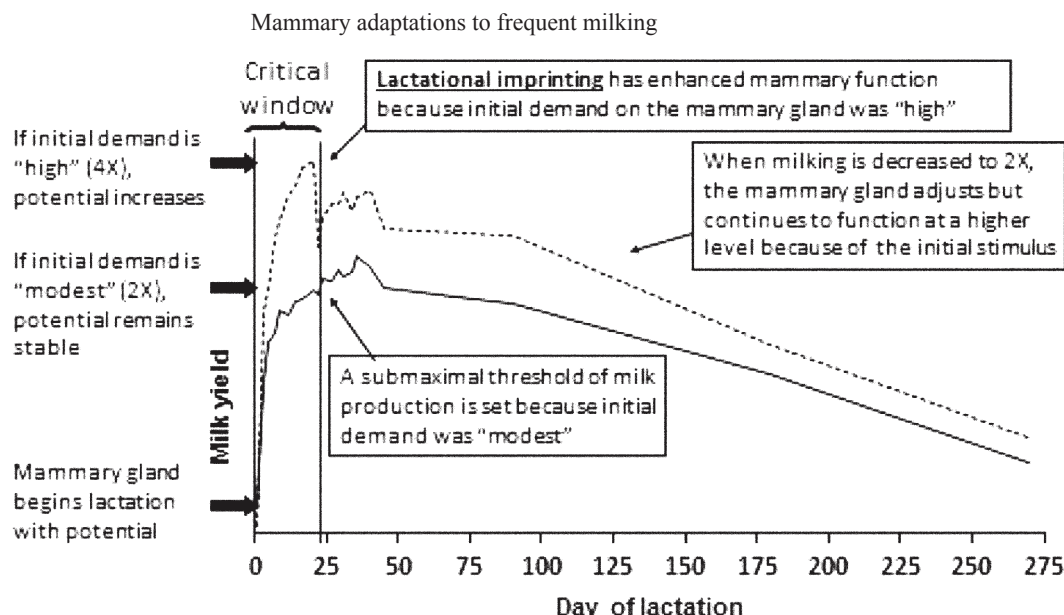
tified contribute to that response? Finally, by what mechanism does IMF during early lactation elicit changes in gene expression that persist long after cessation of treatment?

## LACTATIONAL IMPRINTING: A NOVEL FORM OF AUTOCRINE REGULATION FOR MATCHING MILK SUPPLY TO THE DEMANDS OF THE NEONATE

Imprinting is the process by which cells retain a biological memory of environmental events that occur during critical periods of development. Such events initiate the process of epigenetic regulation, which involves stable, heritable changes in gene expression without changing the DNA sequence itself (Jaenisch and Bird, 2003). Epigenetics is considered a common theme in biology, important for cellular development, differentiation, and memory. The primary mechanisms regulating epigenetic alterations in gene expression are methylation of cytosine residues on the DNA and histone modification (Jaenisch and Bird, 2003). During critical phases of development, imprinting can be initiated by a variety of environmental stimuli, including exposure to hormones or activity of and demand on the tissue or organ (Levenson and Sweatt, 2006). Therefore, imprinting is a mechanism by which environmental stimuli can elicit lasting biological effects long after the cells or tissues were initially exposed to the stimulus.

There is evidence for epigenetic control of gene expression and cellular function in the mammary gland. Plachot and Lelievre (2004) reported that DNA methylation plays a role in mammary cell proliferation and differentiation. Exposure of the virgin rat to the hormones of pregnancy is associated with persistent changes in mammary gene expression, which may be involved in the protective effect of pregnancy against breast cancer (Ginger et al., 2001). In addition, it has been proposed that epigenetic mechanisms underlie acute changes in mammary function and gene expression in dairy cows (Singh et al., 2010).

Based on our observations, we propose that the stimulus of IMF early in lactation results in imprinting of the mammary gland, and thereby alters milk production potential for the remainder of lactation. We have named this imprinting mechanism “lactational imprinting” because gene expression is persistently regulated long after cessation of IMF (Wall et al., 2008). A second integrative model illustrates the hypothetical effects of IMF in early lactation on lactational imprinting and the long-term alteration of mammary function and milk yield (Figure 6). This model is based on our observation that the dynamic milk yield response to 4X milking was associated with differential gene expression and that the temporal pattern of differential gene expression was correlated with differential milk yield. Under our pro-



**Figure 6.** Schematic summary of the effects of frequent milking during early lactation on lactational imprinting and milk production potential.

posed model, the milk production potential of the mammary gland at the beginning of lactation is set, but plastic. Between d 1 and 21 of lactation, there is a critical window of development wherein the mammary gland is receptive to the initial demands of lactation. If initial demand on the gland is “modest” (e.g., 2X milking), the mammary gland perceives this limited demand via a coordinated transcriptional response, and a submaximal threshold of milk yield is set for the remainder of lactation. Alternatively, if initial demand on the mammary gland is “high” (i.e., 4X milking), the stimulus elicits a coordinated transcriptional response of genes involved in the autocrine regulation of milk production. The continued high demand on the mammary gland results in lactational imprinting, which permanently enhances mammary function and leads to increased milk production for the remainder of lactation. After 4X ceases, and demand on the mammary gland declines, there is an acute adjustment in milk production; however, this is followed by stabilization and persistently increased milk production potential for the remainder of lactation. This proposed effect of IMF during early lactation on mammary remodeling is consistent with the concept of “use it or lose it.” If the mammary gland is not “used” to reach its maximum potential at the beginning of lactation, milk production potential for that lactation may be permanently reduced (Wall and McFadden, 2008).

## SUMMARY AND CONCLUSIONS

There are still many unanswered questions regarding the response of the mammary gland to IMF during early lactation. Despite ongoing effort in this area, the cellular mechanisms underlying the milk yield response are not understood. Our investigations have identified a

gene expression signature that is associated with changes in milk yield. Expression of these genes responds to IMF and appears to be partially regulated by lactational imprinting because some of them remain differentially expressed long after cessation of IMF. However, their functional role(s) in the mammary gland remains unclear. Our ongoing experiments are focused on identifying factors involved in the regulation of these genes in the mammary gland and on establishing causal relationships between gene expression, changes in cellular function, and the long-term alteration of milk production potential by lactational imprinting.

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