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Acute milk yield response to frequent milking during early lactation is mediated by genes transiently regulated by milk removal

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Wall EH, Bond JP, McFadden TB. Acute milk yield response to frequent milking during early lactation is mediated by genes transiently regulated by milk removal. *Physiol Genomics* 44: 25–34, 2012. First published October 25, 2011; doi:10.1152/physiolgenomics.00027.2011.—Milking dairy cows four times daily (4×) instead of twice daily (2×) during early lactation stimulates an increase in milk yield that partly persists through late lactation; however, the mechanisms behind this response are unknown. We hypothesized that the acute mammary response to regular milkings would be transient and would involve different genes from those that may be specifically regulated in response to 4×. Nine multiparous cows were assigned at parturition to unilateral frequent milking (UFM; 2× of the left udder half, 4× of the right udder half). Mammary biopsies were obtained from both rear quarters at 5 days in milk (DIM), immediately after 4× glands had been milked (*experiment 1*, $n = 4$ cows), or 2.5 h after both udder halves had last been milked (*experiment 2*, $n = 5$ cows). Affymetrix GeneChip Bovine Genome Arrays were used to measure gene expression. We found 855 genes were differentially expressed in mammary tissue between 2× vs. 4× glands of cows in *experiment 1* (false discovery rate ≤ 0.05), whereas none were differentially expressed in *experiment 2* using the same criterion. We conclude that there is an acute transcriptional response to milk removal, but 4× milking did not elicit differential expression of unique genes. Therefore, there does not appear to be a sustained transcriptional response to 4× milking on *day 5* of lactation. Using a differential expression plot of data from both experiments, as well as qRT-PCR, we identified at least two genes (chitinase 3-like-1 and low-density lipoprotein-related protein-2 that may be responsive to both milk removal and to 4× milking. Therefore, the milk yield response to 4× milking may be mediated by genes that are acutely regulated by removal of milk from the mammary gland.

gene expression; microarray; mammary gland; dairy cows

MILKING DAIRY COWS MORE FREQUENTLY (more than twice daily) during early lactation can be utilized to stimulate an increase in milk production that partly persists through late lactation (5, 14, 22, 58). The mechanisms underlying the milk yield response are unknown; however, we recently reported that the effect is regulated locally within the mammary gland (56). In addition, several researchers have investigated the effects of increased milking frequency on mammary cell proliferation and apoptosis (22, 42, 54) and mammary gene expression (12, 54, 55). Although an increase in the number of mammary secretory cells could explain the long-term increase in milk yield, reports have not shown consistent effects on rates of cellular proliferation or apoptosis. Likewise, although there is clearly a transcriptional response to increased milking frequency, the functional implications of this response and how it may regulate either the immediate effect on milk yield or the carryover effect on milk yield, or both, remain unknown.

One of the factors that complicate interpretation of mechanistic experiments is the timing of sampling relative to milking. Although it may be assumed that milk removal and the stimuli associated with the act of milking elicit an acute transcriptional response in the mammary gland, the effect has not been studied. Thus, it is not currently possible to differentiate between acute responses to regular milking and longer-lasting responses that may mediate the stimulatory effect of increased milking frequency. More specifically, it is difficult to choose a sampling time that captures a true response to increased milking frequency, rather than an acute response of the mammary gland to milk removal.

An acute response to stimulus and/or injury has been observed in many tissues, including skeletal muscle (19, 35), lung (20), and bladder (4, 59), and those responses can be distinguished from long-term changes in gene expression that occur during prolonged stimulus or injury. For example, in response to an increase in physiological demand (exercise), skeletal muscle undergoes a transient process of coordinated change in gene expression that precedes enhanced muscular function (19, 24). Similar observations have been made in nervous tissue, and this is referred to as "synaptic plasticity" (32). With respect to the mammary gland, the acute transcriptional response to milk removal has never been characterized.

We recently observed that although 4× milking did not affect mammary cell proliferation, several genes of the insulin-like growth factor (IGF) axis were acutely regulated by removal of milk from the mammary gland (55). The results of that experiment also indicated that there are differences between the acute transcriptional response of the mammary gland to milk removal and the sustained response of the mammary gland to frequent milking. That research was mainly focused on investigating the effects of milk removal or 4× milking on expression of genes of the IGF axis. We subsequently hypothesized that the expression of other genes is also differentially affected by milk removal or by 4× milking.

In the current study, we used a half-udder model and a transcriptomics approach to characterize and compare the acute transcriptional response to milk removal and the sustained transcriptional response to 4× milking in early lactation dairy cows. We report a marked acute transcriptional response to milk removal, and that some of these genes may mediate the response to 4× milking.

MATERIALS AND METHODS

Animals and Treatments

Experiment 1. Cows used in this study were a subset ($n = 4$) of animals from two larger studies, and details on animal management

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and treatments have been described (56, 57). In brief, Holstein cows (2nd or 3rd lactation) were assigned at parturition to unilateral frequent milking (UFM; 2× milking of the left udder half, 4× milking of the right udder half) beginning on *day 1* of lactation. Regular milkings took place at 0230 and 1430, and the two extra milkings (during which only the right udder half was milked) took place at 0530 and 1730.

Experiment 2. Five multiparous Holstein cows (2nd or 3rd lactation) were assigned to UFM beginning on *day 1* of lactation. Animal management and milking routine were as described for *experiment 1*. One animal was removed from the experiment due to intramammary infection.

Based on our previous power calculations, with as few as three UFM cows, we have 90% power to detect a twofold difference in gene expression and 80% power to detect a 1.6-fold difference in gene expression. We therefore had adequate control over Type II error in the current experiments. Nevertheless, it is possible that some subtle differences in gene expression could be identified with a larger number of animals. For both experiments, the University of Vermont Institutional Animal Care and Use Committee approved animal use and associated procedures.

Mammary Biopsy

Our previous observations (54) and those of Hale et al. (22) indicated that a cellular response to frequent milking could be detected by *day 5* of lactation. In addition, differential milk yield had already reached statistical significance by that time (56). Therefore, to identify early changes in transcription in the current experiments, we chose to obtain mammary biopsies on *day 5*.

Experiment 1. Mammary tissue from the middle of each rear quarter was obtained by biopsy using the method of Farr et al. (17) as described by Wall et al. (55). Biopsies were performed immediately after the 0530 milking (during which only the 4× udder half was milked; Fig. 1). In brief, prior to mammary biopsy, cows were administered an epidural injection containing a cocktail of 7 ml of 2% lidocaine [0.22 mg/kg body weight (BW); Phoenix Pharmaceuticals, St. Joseph, MO] and 1.4 ml of 20 mg/ml xylazine (0.05 mg/kg BW, Phoenix Pharmaceuticals). In addition, just prior to biopsy, lidocaine (3 ml, 0.07 mg/kg BW) was administered in a line-block directly above the incision site. A biopsy sample of ~500 mg (~70 × 40 mm in diameter) was obtained from the middle of each rear quarter. Biopsy samples were trimmed of extraparenchymal tissue and a portion (~200 mg) was immediately frozen in liquid nitrogen for subsequent isolation of RNA. The remaining parenchyma (~5 mg) was diced into pieces that were fixed in 10% buffered formalin for subsequent histological analysis.

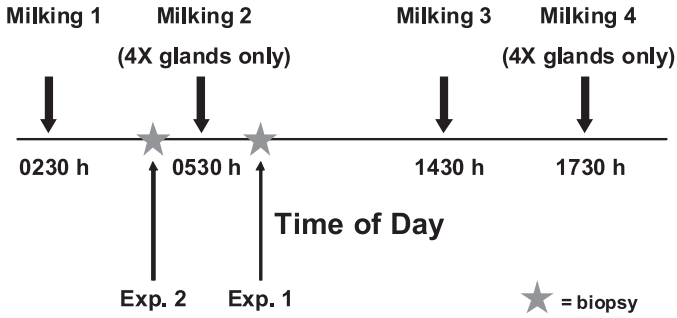

Fig. 1. Milking routine and timing of mammary biopsies. In *experiment 1* (Exp. 1), mammary biopsies were performed immediately after the 4× udder half had been milked but 3.5 h after the 2× udder half had last been milked. In *experiment 2* (Exp. 2), mammary biopsies were performed 2.5 h after both udder halves had been milked. Therefore, differences between udder halves observed in *experiment 1* were classified as an acute mammary response to milking and possible response to 4×, whereas differences between udder halves observed in *experiment 2* were classified as a sustained mammary response to 4× milking treatment.

Experiment 2. Mammary tissue from the middle of each rear quarter was obtained by needle biopsy on *day 5* of lactation as described by Wall and McFadden (55). Biopsies were performed at 0500, ~2.5 h after both udder halves had last been milked (Fig. 1). In brief, prior to mammary biopsy, cows were administered an intravenous injection of 0.50 ml of 20 mg/ml xylazine (0.01 mg/kg BW, Phoenix Pharmaceuticals) for general sedation. In addition, lidocaine (3 ml; 0.07 mg/kg BW) was administered in a line-block directly above the incision site just prior to biopsy. A biopsy sample of ~15–20 mg was obtained using a Pro-Mag Ultra automatic biopsy instrument (Medical Device Technologies, Gainesville, FL). Biopsy of the same site was repeated (~3 times) until ~60 mg of tissue was obtained. Biopsy samples were trimmed of extraparenchymal tissue and a portion (~50 mg) was immediately frozen in liquid nitrogen for subsequent isolation of RNA. The remaining parenchyma (~5 mg) was diced into pieces that were fixed in 10% buffered formalin for subsequent histological analysis.

Interpretation of the Mammary Response to Treatment

In *experiment 1*, mammary biopsies were performed immediately after only the 4× udder halves were milked. In contrast, mammary samples from cows in *experiment 2* were obtained 2.5 h after both udder halves were last milked. Therefore, differences between udder halves observed in *experiment 1* were interpreted as an acute mammary response to milking, or a response to 4× milking, whereas differences between udder halves observed in *experiment 2* were classified as a specific mammary response to 4× milking.

RNA Isolation

Total RNA was isolated from each biopsy sample using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA was purified using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Purified RNA was quantified using a Nanodrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, DE), and quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The RNA integrity number of all samples was >8, and the average 260/280 ratio was 1.99 ± 0.02. Additional details on RNA processing can be found in Wall et al. (52).

Affymetrix GeneChip Bovine Genome Arrays

Microarray analysis. RNA amplification and microarray analysis for both experiments were performed at the University of Vermont microarray core facility using previously described protocols (1). In brief, 2 μg of total RNA from each tissue sample were reverse transcribed to the single-stranded cDNA using T7-oligo(dT) primer. T4 DNA polymerase was used to synthesize double-stranded cDNA, which served as a template for in vitro transcription using T7 RNA polymerase to produce biotinylated cRNA. The biotinylated cRNAs were fragmented into 50- to 200-base fragments and then hybridized to GeneChip Bovine Genome Arrays for 16 h at 45°C in a rotating Affymetrix GeneChip Hybridization Oven 320. After hybridization, arrays were washed and stained with streptavidin-phycoerythrin on an automated Affymetrix GeneChip Fluidic Station F450. The arrays were scanned with an Affymetrix GeneChip Scanner 2700, and the images quantified using Affymetrix GeneChip Operating Software. Sample from one cow in *experiment 2* was eliminated because of inferior RNA quality. Therefore, microarray analysis was performed on mammary samples from four cows in *experiment 1* and three cows in *experiment 2*.

Data analysis. The signal intensity for each probe on each chip was calculated from scanned images using GeneChip Operating Software (Affymetrix), and signal intensities were analyzed using BioConduc-

tor (http://www.bioconductor.org). Probe intensities were background corrected, normalized, and summarized using the Robust Multichip Average method described by Speed and coworkers (8, 25). An alternative normalization method based on housekeeping genes did not significantly change the results. All microarray data were deposited into National Center for Biotechnology Information's Gene Expression Omnibus (accession number GSE27380).

The false discovery rate (FDR) for each individual gene was calculated using the method of Benjamini and Hochberg (see Ref. 6). Gene expression data of each experiment were analyzed using a threshold of FDR $\leq$ 0.05 to identify differentially expressed genes.

Ingenuity Pathway Analysis (IPA; Ingenuity Systems, http://www.ingenuity.com) was used to determine the functions enriched by differentially expressed genes in *experiment 1*. The Affymetrix Bovine Array was used as a reference for the analysis, and all other options used were default settings in IPA. Significance of enriched functions was adjusted for multiple comparisons using the method of Benjamini and Hochberg (6). Using the criterion of FDR $\leq$ 0.05, we found that no genes were differentially expressed in *experiment 2*. However, because *experiment 2* was designed specifically to distinguish potentially subtle changes in gene expression associated with the response to 4× milking from more marked responses to milk removal, we reanalyzed the data using the results of *experiment 1* as a filter. Furthermore, because our goal was to identify transcriptional changes associated with the ~20% difference in milk yield between 2× and 4× udder halves, we set a threshold of similar magnitude for change in gene expression. Hence, the reanalysis included only the 863 probe sets (855 genes) that were differentially expressed in *experiment 1*. For each probe set differentially expressed in *experiment 1*, the $\log_2$ fold change was plotted on the ordinate against the $\log_2$ fold change in *experiment 2*, plotted on the abscissa, to identify genes for which differential expression was correlated across the two experiments. Genes that passed the threshold of $\log_2$ signed fold change $\geq$ 0.40 (or 1.3-fold change) were considered to be putative responders to 4× milking.

It was also of interest to determine which genes would be identified as differentially expressed if we combined the data from both experiments (ignoring the designed difference in sampling times), thereby achieving $n = 7$ cows under comparison. To do so, data for the 855 genes were combined across both experiments and subjected to analysis using a relatively conservative two-tailed paired *t*-test (version 9.1; SAS Institute, Cary, NC) to determine the significance of differential expression of each gene across both experiments.

Real-time Quantitative PCR

Relative mRNA expression profiles of two genes were determined in both experiments by real-time quantitative PCR (qPCR) using a PE 7700 thermal cycler (Applied Biosystems, Foster City, CA). Additional details on PCR can be found in Wall et al. (52). Primers were designed to bovine chitinase 3-like (CHI3L)-1 and low-density lipoprotein-related protein (LRP)-2 using Primer 3 software (http://frodo.wi.mit.edu/primer3/). Primer sequences for CHI3L-1 were forward: 5′-tgggaggagctacactctgg-3′, reverse: 5′-ggcacttgtgagaggaaagg-3′. For LRP-2, primer sequences were forward: 5′-cgtcacagtgtgtgatgcag-3′, reverse: 5′-gcccaaaaccagttactcca-3′. After primer design, the predicted product was BLAST-searched against the bovine database to ensure specificity of the primers. All gene expression values were normalized to that of β-2 microglobulin, which was not affected by UFM ($P >$ 0.40). It is important to note that the use of one reference gene for normalization of qPCR data is not always ideal. For example, Vandesompele et al. (50) suggested that the use of a "normalization factor," calculated from the expression of multiple reference genes, is a more accurate approach to normalizing qPCR data since expression of some reference genes is not always stable. In some cases, however, the use of multiple reference genes is not practical or totally necessary, especially when expression of the chosen reference gene is stable (10, 50). In the current study, because expression of β-2 microglobulin was stable, and we were quantifying the expression of only two genes of interest, we chose to use one reference gene to normalize the qPCR data. For both experiments, a two-tailed paired *t*-test (version 9.1, SAS Institute) was used to determine the significance of treatment effects on mammary gene expression measured by qPCR.

RESULTS

The results for milk yield, mammary cell proliferation, and apoptosis have been reported (55). In brief, after 3 days of UFM and just prior to mammary biopsy, cows in *experiment 1* were producing 12.7 ± 1.2 kg/day, and differential milk production was 3.5 ± 1.0 kg/day more milk from the 4× udder half than the 2× udder half (Supplemental Table S1).[1] Cows in *experiment 2* were producing 13.5 ± 1.9 kg/day, and differential milk production was 2.9 ± 0.6 kg/day more milk from the 4× udder half than the 2× udder half (Supplemental Table S1). Unilateral frequent milking had no effect on mammary cell proliferation or apoptosis (55 and Supplemental Table S1), nor on proportions of epithelial or stromal cells in mammary tissue (70.4 ± 2.6 vs. 65.3 ± 2.6% epithelium and 29.6 ± 2.6 vs. 34.7 ± 2.6% stroma, in 2× and 4× udder halves, respectively).

Microarray Experiments

Experiment 1. The results of our microarray experiment revealed that 855 genes (863 probe sets) were differentially expressed in 4× vs. 2× udder halves of cows in *experiment 1* (FDR $\leq$ 0.05, Supplemental Table S2). We found 199 genes were upregulated in response to 4×, whereas 656 were downregulated; 348 of the differentially expressed genes were nonannotated transcribed loci. Seven genes were represented by multiple probe sets. In those cases, the direction and magnitude of differential expression closely agreed across probe sets.

Experiment 2. Analysis of microarray data revealed that no genes were differentially expressed in 4× vs. 2× udder halves of cows in *experiment 2*, based on a threshold of FDR $\leq$ 0.05 (Supplemental Table S2). As previously stated, because *experiment 2* was designed specifically to distinguish potentially subtle changes in gene expression associated with the response to 4× milking from more marked responses to milk removal, we reanalyzed the data using the results of *experiment 1* as a filter. Hence, the reanalysis included only the 855 genes (863 probe sets) that were differentially expressed in *experiment 1*. Relative expression of those genes in both experiments is illustrated by scatterplot (Fig. 2), and we used this to choose genes that might have been responsive to 4× milking. The differential expression of two of those genes was confirmed by real-time quantitative RT-PCR (Fig. 3).

To further assess differential gene expression, data from both experiments were combined and subjected to analysis using a paired *t*-test. This effectively gave a comparison of 4× vs. 2× milking in udder halves of seven cows. Although conservative, this approach confirmed 26/863 probe sets were differentially expressed at FDR $\leq$ 0.20 and 780/863 probe sets were differentially expressed at $P \leq 0.05$ (Supplemental Table S2).

[1] The online version of this article contains supplemental material.

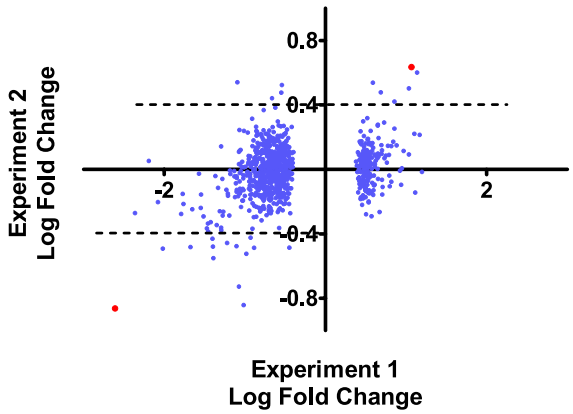

Fig. 2. Scatterplot of genes differentially expressed in response to milk removal or to 4 times daily milking of lactating dairy cows. Cows were assigned to unilateral frequent milking [UFM; twice daily milking of the left udder half (2×), four times daily milking of the right udder half (4×)] starting on *day 1* of lactation. Mammary biopsies from both rear quarters were obtained on *day 5* of lactation. In *experiment 1*, biopsies were obtained immediately after the 4× udder half was milked but 3.5 h after the 2× udder half had last been milked ($n = 4$ cows). In *experiment 2*, biopsies were obtained 2.5 h after both udder halves had last been milked ($n = 3$ cows). There were 863 probe sets differentially expressed in *experiment 1* (FDR ≤ 0.05). For each probe set differentially expressed in *experiment 1*, the $\log_2$ fold change was plotted on the ordinate against the $\log_2$ fold change in *experiment 2*, plotted on the abscissa, to identify genes for which differential expression was correlated across the two experiments. Each data point represents the $\log_2$ fold change (4× vs. 2×) for each probe set. Dashed horizontal lines indicate an arbitrary threshold of differential expression in *experiment 2* (set at $\log_2$ fold change of 0.4- or 1.3-fold change; genes that passed this threshold were considered to be potential candidates that might be specifically responsive to 4× milking). Red data points indicate genes that were confirmed by qPCR.

Functional Analysis

IPA was used to identify functions enriched by differentially expressed genes. Of the differentially expressed genes, 522 were mapped and 418 were eligible for functional and pathway analysis. Significantly enriched functions (Fig. 4 and Supplemental Table S3) and pathways (Supplemental Table S4) were determined. The functions associated with genes differentially expressed in *experiment 1* were cell death, cell growth and proliferation, hematological system development, and tissue morphology. Using the effect on function tool in IPA, we determined that the effect of 4× vs. 2× milking on mammary gene expression was associated with a predicted decrease in cell death, indicated by lower expression of 16 genes known to increase cellular apoptosis including: B-cell translocation gene, antiproliferative (BTG1), CHI3L-1, FBJ murine osteosarcoma viral oncogene homolog (FOS), IGF binding protein (IGFBP)-3, and signal transducer and activator of transcription (STAT)-3. An increase in cell growth and proliferation was predicted by altered expression of 106 genes including: higher expression of pim-1 oncogene (PIM1), platelet-derived growth factor receptor alpha (PDGFRA), and myostatin (MSTN), and lower expression of Krüppel-like factor (KLF)-10. The effect of 4× milking on gene expression and cellular development was predicted to be associated with greater cellular differentiation as indicated by lower expression of fibroblast growth factor receptor (FGFR)-2, keratin (KRT)-8, and lactoferrin (LTF). Finally, changes in tissue morphology were predicted by altered expression of 73 genes including lower expression of early growth response (EGR)-1 and jun B proto-oncogene (JUNB), and higher expression of c-abl oncogene 1 (ABL1). Although no canonical pathways were significantly enriched based on *P* values adjusted for multiple hypothesis testing (Supplemental Table S4), the most affected pathway was PI3K signaling in T lymphocytes, which included 11 genes that were downregulated in mammary tissue from 4× relative to 2× udder halves (Fig. 5 and Supplemental Table S4). This result points to PI3K signaling as a possible mediator of some effects of increased milking frequency and is worthy of further exploration.

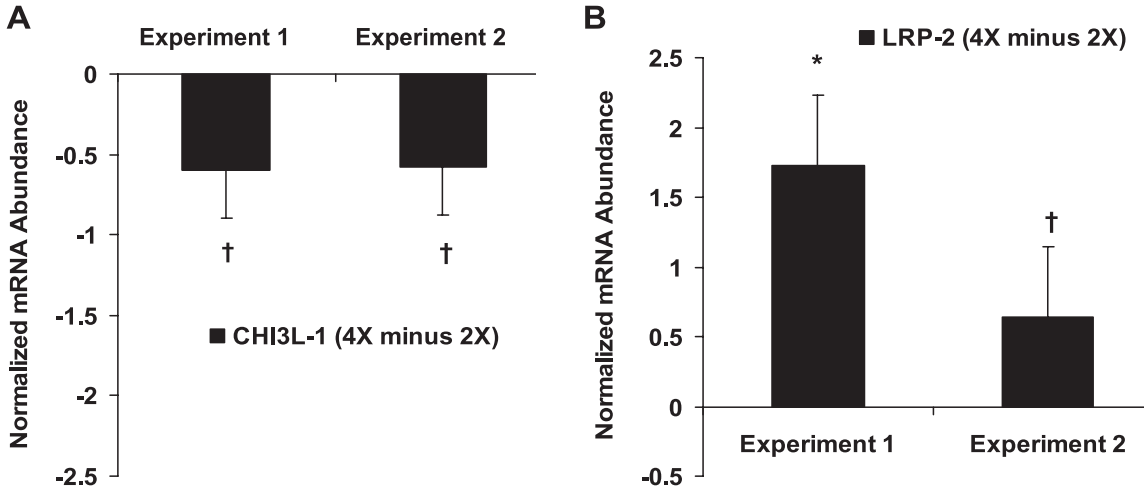

Fig. 3. Examples of genes differentially regulated by milk removal or frequent milking. Expression of chitinase 3-like (CHI3L-1; *A*), and low-density lipoprotein-related protein (LRP-2; *B*) mRNA in lactating bovine mammary gland. Cows were assigned to UFM [twice daily milking of the left udder half (2×), four times daily milking of the right udder half (4×)] starting on *day 1* of lactation. Mammary biopsies from both rear quarters were obtained on *day 5* of lactation. In *experiment 1*, biopsies were obtained immediately after the 4× udder half was milked but 3.5 h after the 2× udder half had last been milked ($n = 4$ cows). In *experiment 2*, biopsies were obtained 2.5 h after both udder halves had last been milked ($n = 4$ cows). Each bar represents the least squares mean difference (4× − 2×) ± pooled SE expression of mRNA normalized to β-2 microglobulin, which was not different between udder halves ($P \geq 0.40$). In *experiment 1*, expression of CHI3L-1 was lower ($P \leq 0.08$), whereas expression of LRP-2 was higher ($P \leq 0.03$) in 4× than 2× udder halves. In *experiment 2*, expression of CHI3L-1 ($P \leq 0.06$) was lower, whereas expression of LRP-2 was higher ($P \leq 0.06$) in 4× than 2× udder halves. *$P < 0.05$; †$P < 0.1$.

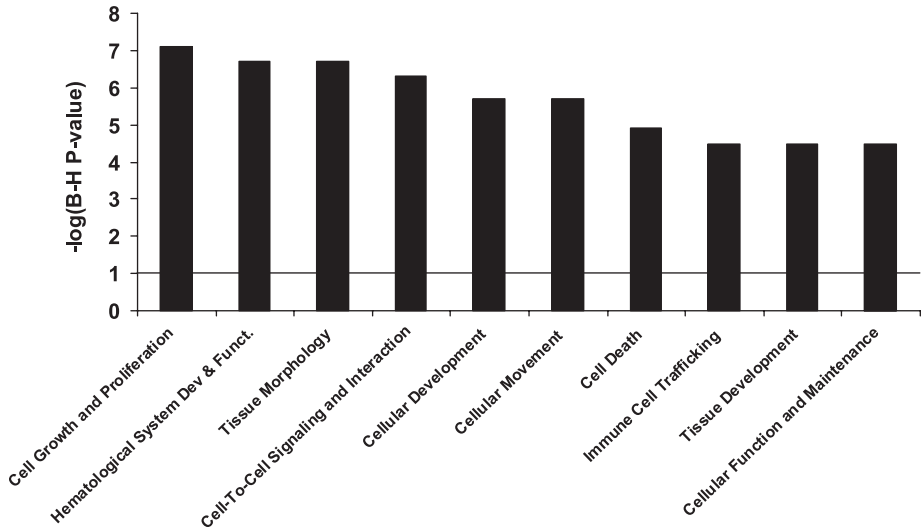

Fig. 4. Ingenuity Pathway Analysis (IPA) of genes differentially expressed in response to milk removal or to 4× daily milking of lactating dairy cows. Cows were assigned to UFM [twice daily milking of the left udder half (2×), four times daily milking of the right udder half (4×)] starting on *day 1* of lactation. Mammary biopsies from both rear quarters were obtained on *day 5* of lactation, immediately after the 4× udder half was milked but 3.5 h after the 2× udder half had last been milked ($n = 4$ cows). Gene expression was detected using Affymetrix GeneChip Bovine Genome Arrays. Differentially expressed genes (FDR ≤ 0.05) were uploaded into IPA, and functional categories were determined. Each bar represents the $-\log$ (B-H P value) for the function listed on the x-axis. The thin horizontal line indicates the FDR cutoff (0.20) for functional significance.

Pathway analysis was not run on *experiment 2* because no genes passed the FDR criterion for differential expression. Nevertheless, one of the most significant networks revealed by pathway analysis of the differentially expressed genes in *experiment 1* (Fig. 6) featured interactions among three genes: CHI3L-1, low density lipoprotein-related protein (LRP)-2, and MSTN, which were provisionally identified as differentially expressed in *experiment 2* based on a threshold of $\log_2$ signed fold change of 0.40 (signed fold change of 1.3; Fig. 2). Because the pattern of expression of two of those genes was similar in both experiments, as confirmed by qRT-PCR (Fig. 3, *A* and *B*), we conclude that they are differentially regulated by UFM and propose that they may mediate the milk yield response to 4× milking as well as to acute milk removal.

DISCUSSION

The response of tissues to physiological stimuli can be classified as acute (short term) or chronic (long term). The distinction between acute and chronic responses of many tissues, including bladder (4, 59), lung (20), nervous tissue (32, 48), and skeletal muscle (19, 35) has been actively studied, and the functional implications of the distinct responses are still under investigation. In the mammary gland, acute responses might be induced by discrete events such as mammary infection, milk stasis/milk removal, or short-term hormone treatment. Chronic or long-term responses might be induced by changes that are imposed repeatedly over time, such as changes in diet, photoperiod, or milking frequency. These may be thought of as adaptive responses. Although several researchers have reported on the transcriptional response of the mammary gland to milk accumulation (53), once daily milking (33), 4× milking (12), involution (31, 45), lactogenesis (18, 31), and photoperiod (51, 52), the acute transcriptional response to milk removal has not been reported. In addition, in previous studies, the "acute" response of the mammary gland to once daily milking, cessation of milking, or resumption of milking has been defined as >6 h after the stimulus was imposed (33, 45, 53).

The objectives of this study were to first identify genes involved in the acute (<30 min) transcriptional response to milk removal and then to distinguish them from a unique set of genes that we hypothesized may specifically regulate the rapid milk yield response to 4× milking during early lactation. The results clearly show a robust, but transient, acute transcriptional response of the mammary gland to regular milk removal. In contrast, we were unable to identify any genes that uniquely responded to 4× milking. This implies that the genes responsible for the milk yield response to 4× are among those that respond acutely to regular milk removal, after only 5 days of UFM immediately postpartum. That is perhaps not surprising because milk yield normally increases rapidly during early lactation, and regular milk removal is necessary to realize that potential. Milking more frequently may simply accentuate those ongoing changes.

Based on microarray analysis, 855 genes were acutely and transiently regulated by milk removal (differentially expressed in *experiment 1* only). That no genes passed the cutoff of FDR $\leq$ 0.05 in *experiment 2* might relate to the relatively short duration of 4× milking (*day 5* of UFM). As mentioned above, however, we previously detected a cellular response to 4× milking by *day 5* of lactation and milk yield of 4× udder halves had increased by 15–20% relative to 2× halves by that time. Nevertheless, because milk yield of both udder halves was increasing at this early stage of lactation, we recognized it might not be possible to distinguish the transcriptional changes that regulate the normal increase in milk yield from those controlling the additional response to 4× milking if no unique genes were involved. It is also possible that changes in expression of some genes did not reach significance based on the stringent statistical cutoffs required for microarray analysis. To address this point, we reanalyzed *experiment 2* to identify genes for which differential expression in *experiment 2* agreed with that in *experiment 1*. Based on a threshold of $\log_2$ signed fold change $\geq$ 0.40 (signed fold change of 1.3; Fig. 2), we identified genes that may have been responsive to 4× milking. For some of these genes, it was clear that they also responded to milk removal since differential expression was greater in *experiment 1* than *experiment 2*. It seems probable that some of the genes that respond to regular milk removal, which is necessary to maintain lactation, might also be involved in the stimulatory response to increased milking frequency.

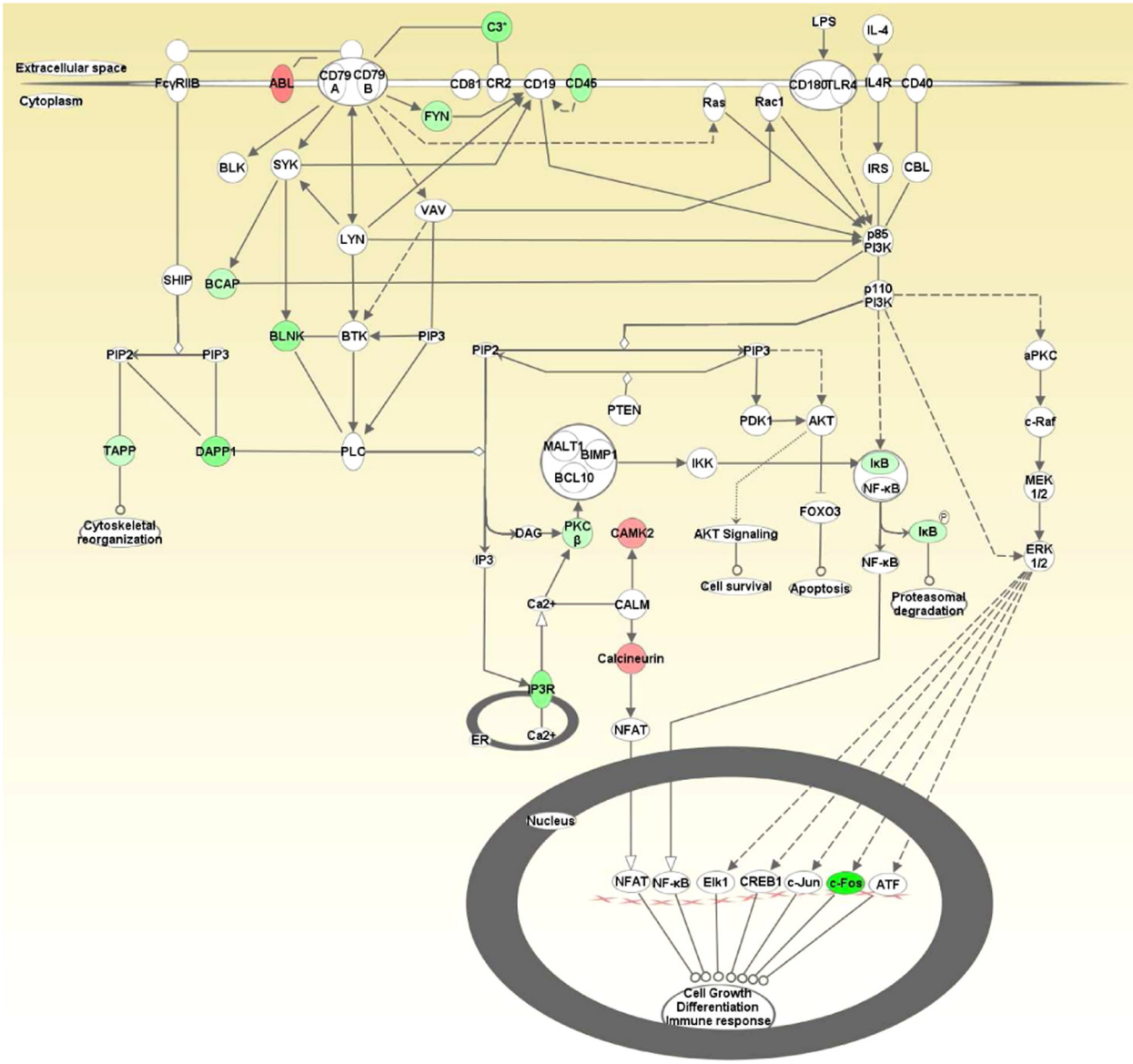

Fig. 5. IPA illustrating the predicted effect of milk removal on PI3K signaling in T lymphocytes. Cows were assigned to UFM [twice daily milking of the left udder half (2×), four times daily milking of the right udder half (4×)] starting on *day 1* of lactation. Mammary biopsies from both rear quarters were obtained on *day 5* of lactation, immediately after the 4× udder half was milked but 3.5 h after the 2× udder half had last been milked ($n = 4$ cows). Gene expression was detected using Affymetrix GeneChip Bovine Genome Arrays. Differentially expressed genes (FDR ≤ 0.05) were uploaded into IPA, and core analysis was run to identify enriched networks. The most affected canonical pathway was PI3 signaling in T lymphocytes. Genes in green were downregulated in mammary tissue from 4× udder halves; genes in red were upregulated.

Acute Response: Genes and Functions

Based on pathway analysis, differentially expressed genes were grouped into functional categories to provide insight into the possible mechanisms involved in the mammary response to milk removal or 4× milking (Fig. 4). Among the affected functions, the results of pathway analysis predicted an increase in cell growth and proliferation, a decrease in cell death, and an increase in cellular development (differentiation). The genes associated with this acute response may be early genes involved in the immediate response of the mammary gland to physiological stimuli. For example, an increase in expression of MSTN and a decrease in expression of KLF-10 in mammary tissue of 4× udder halves was predicted by IPA to be associated with an increase in cell growth and proliferation, as well as cell differentiation. It is well known that MSTN, a member of the transforming growth factor (TGF)-β superfamily, is a key regulator of muscle development and, in muscle, MSTN gene expression is downregulated within minutes of acute bouts of exercise (35). It is less well-known that MSTN, which is also known as growth and differentiation factor-8 (GDF-8), has been previously associated with differentiation of the mammary gland in the mouse (37). In addition to MSTN, KLF-10 is involved in TGF-β signaling and is an established early response gene (2, 13, 27); therefore, it may be similarly regulated and acutely responsive to stimuli in the mammary gland.

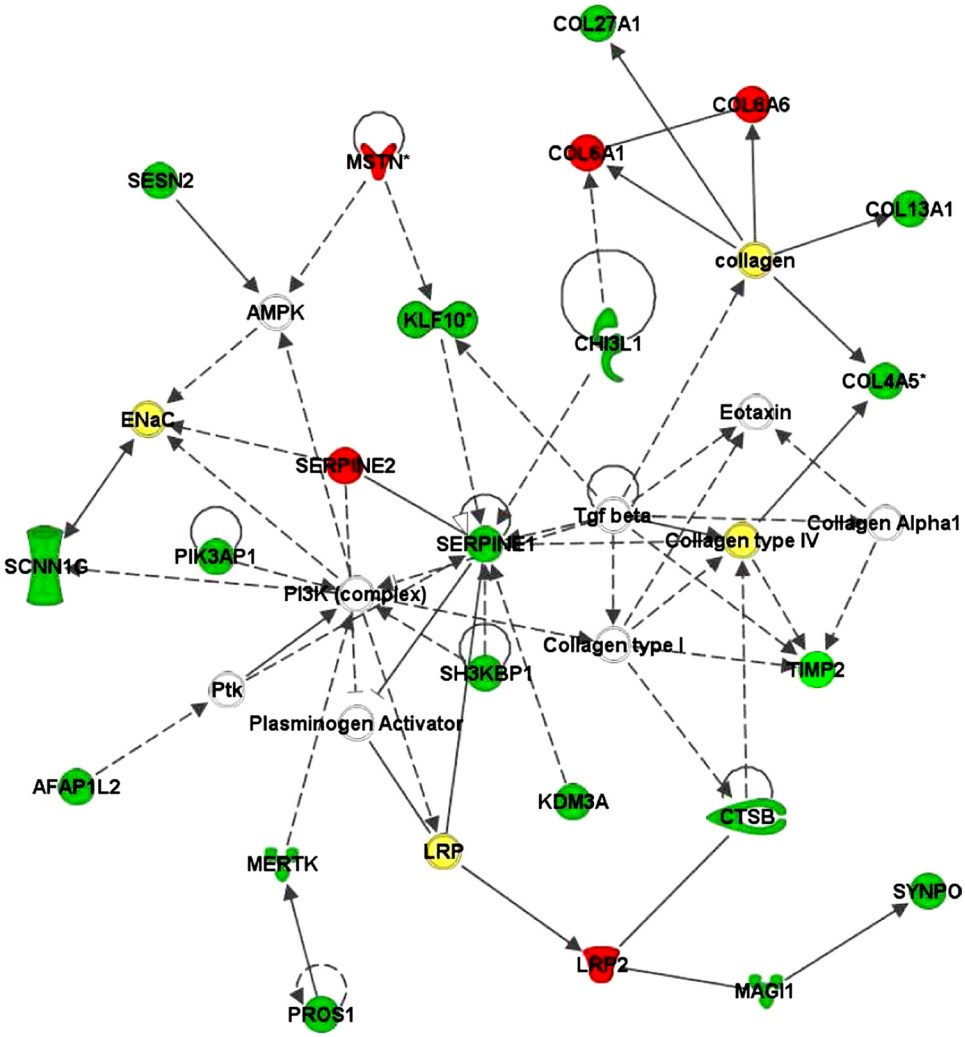

Fig. 6. Ingenuity Network Analysis containing genes differentially expressed in response to milk removal or to 4 times daily milking of lactating dairy cows. Cows were assigned to UFM [twice daily milking of the left udder half (2×), four times daily milking of the right udder half (4×)] starting on *day 1* of lactation. Mammary biopsies from both rear quarters were obtained on *day 5* of lactation, immediately after the 4× udder half was milked but 3.5 h after the 2× udder half had last been milked ($n = 4$ cows). Gene expression was detected using Affymetrix GeneChip Bovine Genome Arrays. Differentially expressed genes (FDR ≤ 0.05) were uploaded into IPA, and core analysis was run to identify enriched networks. Network analysis shows the interaction between genes differentially expressed in *experiment 1* and additional genes that were not differentially expressed. Genes in green were downregulated in mammary tissue from 4× udder halves; genes in red were upregulated; yellow indicates a gene family or group that was enriched by differentially expressed genes. White genes are those that interact but were not differentially expressed.

We previously reported that, in the same tissue used in the current study, expression of IGFBP-1, -3, and -4 was acutely regulated by milk removal (55). In the current study, only IGFBP-3 and cysteine-rich, angiogenic inducer (CYR)-61 (aka IGFBP-10) passed the stringent FDR cutoff; however, in agreement with our previous observations (55), IGFBP-1, -2, and -4 were differentially expressed based on FDR ≤ 0.09. The IGFBP are known to regulate the availability of IGF-I, which has been shown to stimulate cell proliferation and milk yield and is generally considered to mediate the stimulatory effects of bovine somatotropin in the mammary gland (9, 21). Other genes involved in the acute response to milk removal that were downregulated in mammary tissue of 4× udder halves and were predicted by IPA to be associated with a cell proliferation response included EGR-1, CYR-61 (IGFBP-10), LTF, and tumor-associated calcium signal transducer (TACSTD)-2. These genes have previously been associated with the response of the mammary gland to 4× milking (EGR-1; 12) once daily milking (CYR-61, LTF; 33), milk accumulation (53), and mammary involution (LTF; 45).

It is noteworthy that IPA analyses of gene expression predicted that glands milked 4× would exhibit increased cellular proliferation and reduced apoptosis despite our conclusion, based on direct measurements of [^{3}H]-thymidine incorporation and TUNEL, that neither was affected by treatment (55). A limitation of reliance on pathway analyses to derive insights into function is that the functions and canonical pathways generated from microarray data can be misleading. We suggest that many genes grouped into those functional categories are pleiotropic and probably contribute to cellular functions other than proliferation or apoptosis. For example, as pointed out above, decreased expression of IGFBP was predicted by IPA to be associated with increased cell proliferation; however, those genes are also known to be regulated by bovine somatotropin, which stimulates milk yield without affecting cell proliferation. In support of this interpretation, few of the 522 known genes identified in *experiment 1* belonged to the "classical" gene expression signatures of cell proliferation or apoptosis, which typically feature cyclins and caspases, respectively. Moreover, even the caspases, which have been considered hallmarks of apoptosis, have been shown to participate in nonapoptotic functions (30). The pleiotropic nature of many genes is also supported by the fact that, within IPA, genes are often grouped into opposing functional categories (i.e., predicted to both increase and decrease a function), depending on the cell or tissue in question. Therefore, although pathway analysis can provide insight into functions and how genes might interact to influence a function, it does not replace direct functional

measurements. Rather, it may implicate unforeseen pathways and functions, such as PI3K signaling in the present studies, that can be targeted for confirmation in further experiments.

We are confident that there was no effect of UFM on intramammary infection, because there was no difference between udder halves in milk somatic cell count at the time of biopsy (56, 57). Therefore, the striking inflammatory signature we observed in the acute response to milk removal, characterized by downregulation of genes of the complement system as well as several chemokine ligands (Supplemental Table S2), may be indicative of an acute-phase response associated with changes in intramammary pressure or mammary blood flow. Indeed, both intramammary pressure (43) and mammary blood flow (16) are responsive to milk removal, and changes in expression of genes associated with mammary inflammation are known to be associated with 4× milking (12), once daily milking (33), and milk stasis (45). In addition, expression of EGR-1 is co-regulated with acute-phase response genes during inflammation in the eye (15), liver (36), and lung (39). Moreover, EGR-1 expression is upregulated as part of the mammary response to milk accumulation (53), and it is also upregulated and thought to mediate immediate early changes in blood flow during liver remodeling (41). Therefore, we propose that an acute-phase response involving EGR-1 is induced after as little as 3.5 h of milk accumulation (as seen in the 2× gland), and that this response is downregulated by removal of milk from the gland (as seen in the 4× gland).

Taken together, the genes and functions that were acutely responsive to milk removal indicate that within minutes of milking, the mammary gland undergoes an acute response to adapt to the change in intramammary pressure and/or mammary blood flow or removal of milk components (acute phase/inflammatory response), as well as a possible protective response to prevent cells from undergoing apoptosis and reduce the chance of intramammary infection. These responses may be critical components underlying the importance of regular milk removal for maintaining secretory activity in the gland and sustained milk production during lactation.

Response to 4× Milking: Genes and Functions

The identification of genes that were differentially expressed in both experiments was intended to provide insight into potential underlying mechanisms and cell functions associated with the rapid increase in milk yield that occurs almost immediately after switching to 4× milking (58). However, because no genes passed the FDR threshold of ≤ 0.05, we did not run functional analysis on that experiment. Instead, we used a differential expression plot to identify genes that were differentially expressed in *experiment 1* and exhibited a similar pattern of expression in both experiments; we considered that these may have responded to 4× milking but did not pass our FDR threshold (Fig. 2). Moreover, core IPA revealed that several of these genes are known to interact (Fig. 6). Based on this, several genes were identified as putative responders to 4× milking, and some functional interpretation is possible based on what is known about the genes. Genes that were among the 855 that were differentially expressed in *experiment 1* and passed the threshold of $\log_2$ signed fold change ≥ 0.40 in both experiments (1.3-signed fold change; Fig. 2) included γ-actin (ACTG)-2, glutamate receptor interacting protein (GRIP)-1, and KRT-8. As mentioned previously, pathway analysis predicted that decreased expression of KRT-8 in 4× udder halves would be associated with an increase in cellular differentiation. In support of the IPA results, KRT-8 expression is responsive to decreased milking frequency (33) and is known to be involved in mammary alveolar development and differentiation in mice (34). Expression of LRP-2 was increased in response to 4× milking (Supplemental Table S2 and Figs. 2 and 3), which IPA predicted would be associated with an increase in cell proliferation and tissue development. Indeed, LRP-2 has been implicated in regulation of mammary function, differentiation, and nutrient transport (11, 38). Pathway analysis implicated decreased CHI3L-1 expression in the predicted reduction in cell death in response to 4× milking. In agreement, CHI3L-1, which is a secreted protein, has been previously reported to be upregulated during mammary involution and remodeling in dairy cows (28, 44), goats (29, 40), and sheep (46). More recently, differential expression of CHI3L-1 mRNA was linked to lactogenic changes in mammary cells in culture (47). In other tissues, CHI3L-1 is involved in cellular differentiation and remodeling (3, 7, 26) and in the cellular response to mechanical stimuli and pressure (49, 60, 61). Together, these observations are consistent with the involvement of CHI3L-1 in mediating the response of the mammary gland to the stimulus of 4× milking.

Frequent milking during mid- or late lactation was associated with an increase in mammary cell activity and DNA synthesis (23, 42). Therefore, it is possible that the stimulus of 4× milking during early lactation is associated with an increase in mammary cell activity, via increased activity per cell and/or recruitment and activation of quiescent cells, and that is responsible for the rapid increase in milk yield. Indeed, IPA predicted that, based on the effect of 4× on mammary gene expression, cellular differentiation would be enhanced. This response may also be involved in the carryover effect on milk yield associated with 4× milking during early lactation; however, additional studies over a longer timeframe relative to UFM will be necessary to answer that question.

The results of these experiments clearly show that the transcriptional response of the mammary gland to milk removal is transient and probably involves some of the same genes that respond to 4× milking. Functional analyses revealed that the acute response to milk removal is associated with an acute-phase response and possible protection against infection and apoptosis. Interestingly, this may offer an alternate explanation of why frequent milk removal can enhance recovery from mastitis; an effect that has typically been attributed to "flushing out" of bacteria. Although we were not able to run pathway analysis on genes from *experiment 2*, the genes we identified as putative responders to 4× milking have been associated with regulation of mammary cell activity and nutrient metabolism. We are currently summarizing experiments that involve sequential sampling of mammary tissue at several times during the response to 4× milking. Findings may provide further insight into the potential mechanisms and cellular processes that distinguish the acute, transient response of the mammary gland to milk removal from the specific response of the mammary gland to 4× milking, and the carryover effect of 4× milking during early lactation on persistency of lactation.

We conclude that the acute transcriptional response of the mammary gland to milk removal is transient and is diminished

by 3.5 h after milking. In addition, 4× milking did not elicit the differential expression of any unique genes. Therefore, there does not appear to be a sustained transcriptional response to 4× milking on *day 5* of lactation. Gene expression signatures imply that at least some of the genes responsive to milk removal also respond to 4× milking. Additional experiments are necessary to confirm the response of these genes to 4× milking and to determine the function of the genes associated with each response and their role in the regulation of milk production in lactating dairy cows.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: E.H.W. and T.B.M. conception and design of research; E.H.W. performed experiments; E.H.W. and J.P.B. analyzed data; E.H.W., J.P.B., and T.B.M. interpreted results of experiments; E.H.W. prepared figures; E.H.W. drafted manuscript; E.H.W. and T.B.M. edited and revised manuscript; E.H.W. and T.B.M. approved final version of manuscript.

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