BRIEF COMMUNICATION: The effect of milking frequency in early lactation on gene expression in the bovine mammary gland

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Keywords: next generation sequencing; mRNA-seq; milking frequency; mammary gland; dairy cow

Introduction

In ruminants, altered milking frequency influences milk production due to either an increase in the number of secretory mammary epithelial cells and/or the activity of these cells (Stelwagen 2001). Microarray studies examining the response of mammary epithelial cells to either increased (Connor et al. 2008) or decreased (Littlejohn et al. 2010) milking frequency, reported altered gene expression in several regulatory pathways. These changes would appear to be locally regulated within the mammary gland, as milk production is different in different glands within the same udder depending on the milking frequency of individual glands (Stelwagen 2001). Therefore, the udder lends itself well as a model system to study the effect of different milking frequencies by comparing the response in adjacent udder halves (Murney et al. 2012).

The aim of this study was to use this model for mRNA-seq analysis to identify genes and cell signalling pathways involved in regulating milk production. This information may allow the development of novel strategies for improving milk production, especially during reduced milking frequency.

Materials and methods

Animals and manipulations

The experimental design has been described previously (Murney et al. 2012). Briefly, 17 multiparous Holstein-Friesian and Holstein-Friesian X Jersey cows were milked twice daily (2X) until the experiment commenced two to seven days in milk. Udders halves were milked either once a day (1X) at 1100 hours or four times a day (4X) at 1100, 1700, 2300 and 0500 hours for 14 days. For the entire treatment period cows were grazed on pasture *ad libitum* and supplemented with two kg of a commercial concentrate (12.9 MJ/kg metabolisable energy and 12.0% crude protein based on dry matter) per day. On Day 14, three to five hours after the 1100 hours milking both rear quarters of ten randomly selected cows were biopsied as described by Farr et al. (1996).

RNA extraction and next generation sequencing

Total RNA from both udder halves of four cows with the most extreme casein expression differences was extracted and purified as described by Singh et al. (2005). The RNA was then sent to Macrogen Inc, (Seoul, Korea) for sequencing, using an Illumina Hiseq 2000 machine, yielding between 32 million and 42 million 100bp single end reads per sample.

Mapping and analysis of data

Regions of low quality sequence and Illumina primers and adapters remaining from the sequencing process were removed from the reads. These reads were mapped against the bovine RefSeq RNAs, which included both coding and non-coding RNAs, using BWA (Li & Dubin 2009). Uniquely mapped reads, on average, 70% of all reads, were summed for each gene within each sample for statistical analysis.

Statistical analysis

The summed counts were loaded into the statistical software R (R Development Core Team 2012) and analysed using the "edgeR" package (Robinson et al. 2008). Trimmed mean of M normalisation (Robinson & Oshlack 2010) was used to account for the differences in sample size and relative levels of gene expression between replicates. Following normalisation, the counts were modelled using a negative binomial distribution and the differential gene expression between 1X and 4X a day milking was calculated using Fisher's exact test. Genes with fold-changes greater than 1.5 and False Discovery Rate (FDR) corrected P values of around, or less than 0.05, were considered to be differentially expressed.

Ingenuity pathway analysis

The differential gene expression results were analyzed by 'ingenuity pathway analysis' software to find relevant biological themes and networks. Differentially expressed genes with fold-changes greater than 1.5 and FDR corrected P values of less than 0.05 were chosen for the analysis.

Results and discussion

Of the ten most highly expressed genes for all samples, seven encoded milk proteins (Table 1). In the 4X milked samples, these seven genes accounted for around 78% of the reads mapped against the National Center for Biotechnology Information (Bethesda, Maryland, USA) bovine reference sequence RNAs. However, they accounted for around

Table 1 National Center of Biotechnology Information (NCBI) accession number, log_2 fold change and False Discovery Rate (FDR) adjusted P value for the ten most highly expressed genes for all samples. The seven genes encoding milk proteins are indicated.

Gene	NCBI accession number	Log_2 fold change	FDR adjusted P value	Milk protein coding
αs1-Casein (CSN1S1)	NM_181029.2	6.916	1.87E-52	Yes
β-Casein (CSN2)	NM_181008.2	7.173	6.83E-55	Yes
αS2-Casein (CSN1S2)	NM_174528.2	7.089	2.26E-54	Yes
κ-Casein (CSN3)	NM_174294.2	5.294	1.29E-36	Yes
Progestagen-associated endometrial protein (PAEP)	NM_173929.3	7.245	1.27E-55	Yes
α-lactalbumin (LALBA)	NM_174378.2	7.524	6.66E-58	Yes
Glycosylation-dependent cell adhesion molecule 1 (GLYCAM1)	NM_174828.2	6.983	2.19E-53	No
Stearoyl-CoA desaturase (delta-9-desaturase) (SCD)	NM_173959.4	5.836	2.22E-40	No
Lactotransferrin (LTF)	NM_180998.2	-1.941	2.24E-06	Yes
Fatty acid synthase (FASN)	NM_001012669.1	5.618	1.66E-38	No

6% in 1X milked samples. This has implications for detection of less common genes in the 4X a day milking as there are less reads available to detect them, requiring careful normalisation of the samples. Following normalisation, many of the milk protein coding genes and ribosomal protein genes were upregulated in the 4X a day milking, which agrees with the increased milk protein coding gene expression and milk yield reported by Murney et al. (2012).

Transcripts encoding innate immune factors (LBP, SAA3, BCL6, LTF and LAP), and oxidative stress response proteins (SOD2, FTL, SSAT, MT1A and MT2A) were significantly (FDR <0.05) less expressed in the 4X tissue. These results agree with the observation that conversely these processes are upregulated during milk accumulation or early mammary involution (Singh et al. 2005, 2008a). In addition, ingenuity pathway analysis indicated that expression of genes involved in cellular movement, cell proliferation and inflammatory responses was down-regulated in 4X-tissue.

The ingenuity pathway analysis indicated that in 4X tissue, compared to 1X, expression of genes involved in the following immune system pathways were significantly (P <0.05) down-regulated: interleukin-8 signalling, Acute phase response signalling, chemokine (C-X-C motif) receptor 4 signalling, leukocyte extravasation, liver X receptor-retinoic X receptor activation and renin-angiotensin signalling. The acute phase response signalling and the leukocyte extravasation pathways were also upregulated in early stage involution (Singh et al. 2008b), indicating that a reduction in milking frequency may induce a similar physiological state to involution. Indeed, 1X induces a number of gross physiological and cellular changes that represent the onset of mammary involution (Stelwagen, 2001). Although Connor et al (2008) showed changes in mammary gene expression, they compared 2X and 4X mammary glands, whereas the current study used a more 'extreme' comparison, comparing 1X and 4X milking, indicating that also at the level of gene expression, 1X is more representative of the onset of mammary involution. Taken together this study indicates that the unilateral 1X/4X milking regime provides a good model to study changes in mammary gene expression and to develop a bioinformatics pipeline with microRNA-seq, reduced representation bisulphate sequencing, DNAseI-seq for identifying gene candidates.

Acknowledgements

The authors gratefully acknowledge the assistance of science staff from Dairy Science and the farm staff at AgResearch Tokanui Dairy Research Farm and the statistical advice of Harold Henderson. This research was funded by the Ministry of Business, Innovation, and Employment.

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