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The influence of extracellular matrix and prolactin on global gene expression profiles of primary bovine mammary epithelial cells *in vitro*

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Summary

An *in vitro* bovine mammosphere model was characterized for use in lactational biology studies using a functional genomics approach. Primary bovine mammary epithelial cells cultured on a basement membrane, Matrigel, formed three-dimensional alveoli-like structures or mammospheres. Gene expression profiling during mammosphere formation by high-density microarray analysis indicated that mammospheres underwent similar molecular and cellular processes to developing alveoli in the mammary gland. Gene expression profiles indicated that genes involved in milk protein and fat biosynthesis were expressed, however, lactose biosynthesis may have been compromised. Investigation of factors influencing mammosphere formation revealed that extracellular matrix (ECM) was responsible for the initiation of this process and that prolactin (Prl) was necessary for high levels of milk protein expression. *CSN3* (encoding κ-casein) was the most highly expressed casein gene, followed by *CSN1S1* (encoding αS1-casein) and *CSN2* (encoding β-casein). Eighteen Prl-responsive genes were identified, including *CSN1S1*, *SOCS2* and *CSN2*, however, expression of *CSN3* was not significantly increased by Prl and *CSN1S2* was not expressed at detectable levels in mammospheres. A number of novel Prl responsive genes were identified, including ECM components and genes involved in differentiation and apoptosis. This mammosphere model is a useful model system for functional genomics studies of certain aspects of dairy cattle lactation.

Keywords extracellular matrix, gene expression, mammary epithelial cell, prolactin.

Mammary epithelial cells (MEC) are the central component of mammary alveoli, which develop during gestation and are responsible for milk production during lactation. Fully differentiated alveoli are comprised of a single layer of polarized MEC enclosing a lumen, surrounded by a discontinuous layer of myoepithelial cells that allow MEC to contact the external basement membrane; which is a specialized type of extracellular matrix (ECM) (Katz & Streuli 2007). It is generally considered that both the ECM and lactogenic hormones, primarily prolactin (Prl), are required to produce fully functional alveoli.

Extracellular matrix is a regulator of MEC function and MEC adhere to basement membrane via several types of ECM receptors, including the integrins (Katz & Streuli 2007). β1-integrin has an essential role in alveolar development and differentiation, as deletion of the *β1-integrin* gene results in malformed alveoli and failure of Prl-induced MEC differentiation (Naylor *et al.* 2005a). Prl is involved in both MEC proliferation and differentiation during pregnancy and is essential for the secretion of milk into the alveolar lumen in most mammalian species. Prl induces transcription of milk protein genes in the final stages of differentiation via the Jak2-Stat5 pathway. It also induces

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transcription of genes involved in intracellular (keratins) and extracellular structure (laminins and collagens), tight junctions, cell–cell communication and transcription factors involved in differentiation (Ormandy *et al.* 2003). However, Prl only functions in the presence of an appropriate ECM and requires a laminin-rich basement membrane (Katz & Streuli 2007). While there is cross-talk between the ECM and Prl signalling, the contribution of each to MEC differentiation is not fully understood.

We have characterized a bovine mammosphere model consisting of primary bovine MEC (BMEC) cultured on Matrigel, a commercially available ECM, which effectively mimics mammary gland basement membrane (Kleinman & Martin 2005; Katz & Streuli 2007). The contributions of ECM and Prl to mammosphere formation and milk protein expression were assessed by microarray expression profiling and the expression of selected genes was validated by quantitative reverse transcriptase-PCR (qRT-PCR).

Materials and methods

Primary bovine mammary epithelial cell isolation and culture

Mammary biopsies were taken from two non-lactating multiparous Holstein–Friesian cows 30–40 days prior to parturition (Sheehy *et al.* 2004) and BMEC isolated as described previously (Riley *et al.* 2008) to establish two primary BMEC lines. Samples for microarray analysis were produced from each of these cell lines as follows: Passage 1 BMEC were grown in proliferation media (4.75 g/l M199, 5.32 g/l Hams F12, 2.38 g/l HEPES, 1.875 g/l $NaHCO_3$, 0.164 g/l sodium acetate, 20% Horse Serum, 5% FCS, 100 U/ml penicillin/100 μg/ml streptomycin, 100 μg/ml kanamycin, 1.25 μg/ml Fungizone, 5 μg/ml insulin, 1 μg/ml cortisol and 10 ng/ml EGF) to confluence and then harvested with trypsin. Cells (2×10^7) were harvested for RNA extraction to produce the day 0 (D0) sample, while the remainder were plated onto Matrigel (25 μl/cm^2; BD Biosciences) in 6-well plates at 2×10^6 cells/well in attachment media (4.75 g/l M199, 5.32 g/l Hams F12, 2.38 g/l HEPES, 1.875 g/l $NaHCO_3$, 0.164 g/l sodium acetate, 20% horse serum, 5% FCS, 100 U/ml penicillin/100 μg/ml streptomycin, 100 μg/ml kanamycin, 1.25 μg/ml Fungizone, 5 μg/ml insulin, 1 μg/ml cortisol and 10 μg/ml transferrin) containing either no Prl, 0.3 μg/ml Prl or 3 μg/ml Prl (Prl was obtained from the National Hormone & Peptide Program, NIDDK and Dr A Parlow). Cells were incubated at 37 °C, 5% CO_2. After 24 h (day 1) attachment media was changed to differentiation media (4.75 g/l M199, 5.32 g/l Hams F12, 2.38 g/l HEPES, 1.875 g/l $NaHCO_3$, 0.164 g/l sodium acetate, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml kanamycin, 1.25 μg/ml Fungizone, 5 μg/ml insulin, 1 μg/ml cortisol and 10 μg/ml transferrin) again with varying Prl concentrations. Cells were then grown in differentiation media for 4 days (days 2–5). Six wells of each Prl concentration were harvested for RNA extraction on days 1, 2 and 5 (D1, D2 and D5).

RNA extraction, cDNA synthesis and quantitative qRT-PCR analysis

RNA was extracted and cDNA was generated for qRT-PCR analysis as described previously (Riley *et al.* 2008). Expression levels of selected genes for the three Prl concentration levels on D0, D1, D2 and D5 of mammosphere culture were determined by qRT-PCR analysis using gene specific primer sets (Table 1). Each gene was amplified in a separate reaction and each reaction was performed in quadruplicate. Each 10 μl reaction contained 20 ng cDNA for the casein gene analysis, 33 ng for *ELMO1* and *BOC* analysis, 50 ng for *SOCS2* analysis or 10 ng cDNA for the *RPLP0* housekeeping gene analysis, 0.3 μM each primer, 0.2 mM dNTPs, 0.05 U Platinum Taq polymerase (Invitrogen), 1× Platinum Taq buffer, 3 mM $MgCl_2$ and 0.4× SYBR green. For each gene, 0.5 fg to 50 pg of purified PCR product was amplified under the same conditions to generate standard curves from which the copy number was determined. Reactions were incubated at 95 °C for 2 min followed by 35 cycles of 95 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s in a RotorGene 3000 (Corbett Research). Finally, a 72–99 °C gradient at 5 s per degree was performed for a melt curve analysis to confirm amplification of a single product. *RPLP0* results were used to normalize the results for the genes of interest.

Microarray analysis and qRT-PCR validation

Ten RNA samples from each BMEC line were labelled and then hybridized to the Affymetrix Bovine Genome Arrays

Table 1 Primer sets used for qRT-PCR analyses.

Gene symbol and Accession no.	Primer sequence
CSN2 NM_181008	Fwd 5′-TCTGCCTCTGCTCCAGTCTT-3′
	Rev 5′-AGGAGGGGGCATTCACTTT-3′
CSN3 NM_174294	Fwd 5′-CACCCACACCCACATTTATC-3′
	Rev 5′-GACCTGCGTTGTCTTCTTTG-3′
CSN1S1 NM_181029	Fwd 5′-CCTGTCTTGTGGCTGTTGCTC-3′
	Rev 5′-CATCTTCCTTTTGAATGTGCTT-3′
SOCS2 NM_177523	Fwd 5′-GGGACTGCCTTTACCAACAA-3′
	Rev 5′-GTGCTGGGACCTTTCACCTA-3′
ELMO1 NM_001113227	Fwd 5′-CTGTATGTGCTGCAGGTGCT-3′
	Rev 5′-CAGGAGGGGTCTGGGTAAA-3′
BOC XM_867563	Fwd 5′-CGAAGGAGGGGAGAGTGAG-3′
	Rev 5′-ACAAGCAGAAGGGGATGAAG-3′
RPLP0 NM_001012682	Fwd 5′-CAACCCTGAAGTGCTTGA-3′
	Rev 5′-AGGCAGATGGATCAGCCA-3′

according to the manufacturer's recommendations (Australian Genome Research Facility, Melbourne, Australia). Normalization and probe set intensities were calculated using the Robust Multi-array Average method (Iziarry *et al.* 2003) as implemented in the *Affy* package of Bioconductor (Gautier *et al.* 2004). The data were subsequently analysed using the *Limma* package in Bioconductor, to determine genes with differential expression. Multiple testing was addressed using the Benjamini-Hochberg (BH) false discovery rate (FDR) controlling procedure. Complete linkage hierarchical clustering of genes differentially expressed between D1 and D5 was performed in R (http://www.r-project.org/) using the *heatmap.2* function in the *gplots* library. Gene ontologies within clusters were determined using the functional annotation tool in DAVID (http://david.abcc.ncifcrf.gov).

To assess the similarity of result of microarray expression and RT-PCR data, correlation coefficients were obtained between microarray expression values and qRT-PCR values for each gene (on the logarithmic scale). A detailed description of the statistical analysis can be seen in Appendix S1.

Results

Differentiation of primary BMEC to form alveoli-like mammospheres on Matrigel *in vitro* was characterized by gene expression profiling. Primary BMEC cultured on plastic displayed the classic cobblestone morphology (Fig. 1a). During the first 24 h on Matrigel, BMEC attached to the matrix and then migrated to form three-dimensional cell aggregates termed mammospheres (Fig. 1b), regardless of the Prl concentration. Our previous studies have shown that D5 mammospheres have a lumenal space and that maximal casein expression occurs at this time (data not shown), indicating that differentiation has occurred. Rose *et al.* (2002) also reported clearly visible lumen in D5 mammospheres.

After treatment with Prl at levels comparable with those of previous studies (Cirfrian *et al.* 1994; Rose *et al.* 2002), the number of genes differentially expressed between days of culture tended to increase with divergence in culture conditions or duration of culture, and more genes were up-regulated than down-regulated over the culture period (Table 2). Differential gene expression between successive time points was the greatest between D0 and D1, where BMEC were transferred from proliferating to differentiating culture conditions. Similarly, between D1 and D2, where cells were transferred from serum to serum-free media, pronounced changes in gene expression were observed.

Hierarchical clustering of the 3188 genes differentially expressed between D1 and D5 showed that the day of culture rather than Prl concentration was significant (Fig. 2). Gene expression patterns of proliferating BMEC (D0) clustered separately to differentiating BMEC (D1, D2 and D5).

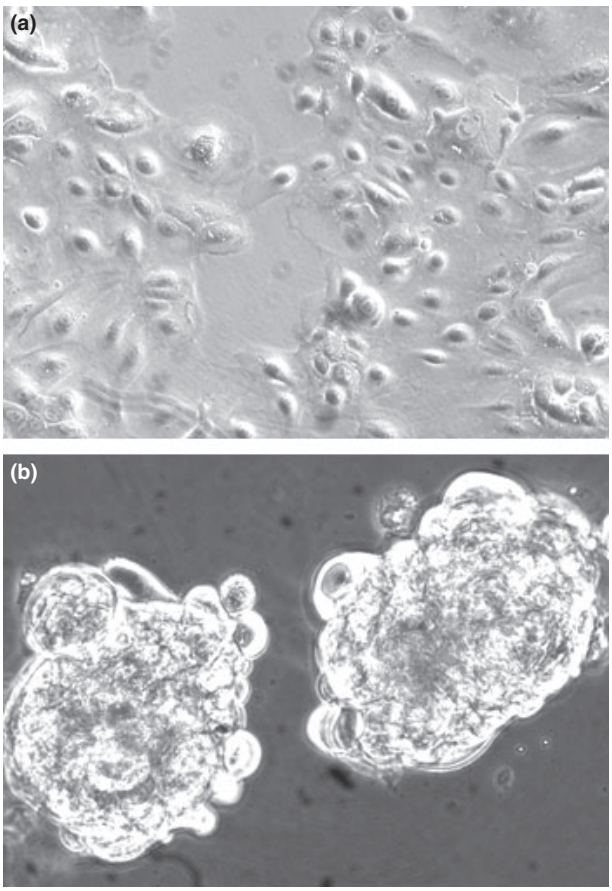

Figure 1 Bright field microscopy of BMEC grown on tissue culture plastic (a) and on extracellular matrix in the presence of lactogenic hormones (b).

Table 2 Number of genes differentially expressed (BH adjusted $P < 0.01$) between days of culture under standard mammosphere culture conditions (i.e. high Prl).

	D0 vs. D1	D1 vs. D2	D2 vs. D5	D1 vs. D5
Up-regulated	762	430	170	1876
Down-regulated	410	231	141	1312
Total	1172	661	311	3188

D1 BMEC on ECM in the presence of serum clustered separately to D2 and D5 BMEC, which were under serum-free conditions. Six gene clusters showing different expression patterns over the culture period were identified (Fig. 2) and ontologies were determined for the genes within each cluster (Table 3).

Genes within cluster 1 were highly expressed on D0 and D1 in culture and down-regulated over subsequent days. Gene ontologies in this cluster included lipid metabolism (adjusted $P < 0.001$), biosynthesis (adjusted $P = 0.066$), sterol synthesis (adjusted $P < 0.001$) and metabolism (adjusted $P = 0.012$), and included genes encoding fatty acid coenzyme-A ligase very long-chain 1, NAD(P)-dependent

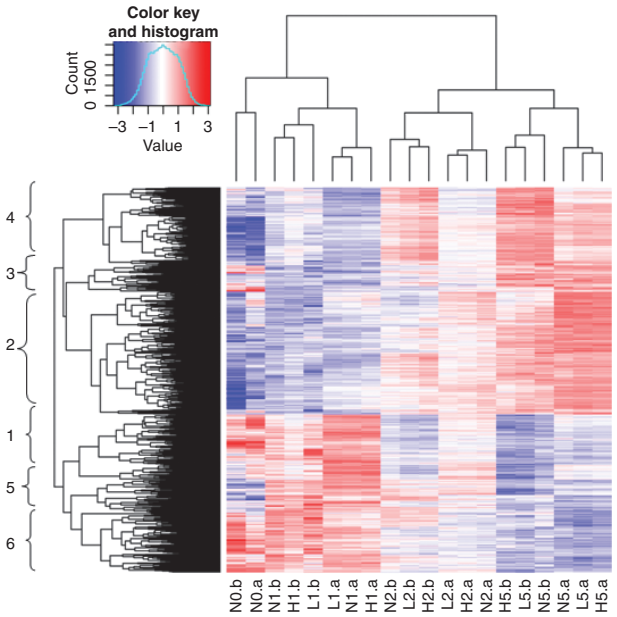

Figure 2 Hierarchical clustering of the 3188 genes differentially expressed between D1 and D5. Treatments are shown as: N, no Prl; L, low (0.3 μg/ml) Prl; H, high (3 μg/ml) Prl; a, BMEC line a; b, BMEC line b; 0, 1, 2 and 5 indicates day of culture.

steroid dehydrogenase-like protein, phosphomevalonate kinase, 7-dehydrocholesterol reductase and *n*-acetylneuraminic acid synthase. Genes from cluster 6 showed a similar expression pattern to genes in cluster 1, however, the level of expression varied between the replicate cell lines in the two clusters. There were no ontologies with a significant adjusted *P*-value in this group, however, genes encoding proteins involved in ECM-receptor interactions, including collagen IIα1 and collagen XIα2, α2-, α3- and β4-integrin and γ1- and γ2-laminin, had the most significant unadjusted *P*-value ($P = 0.0025$). Cluster 5 genes were up-regulated on D1 but this was not sustained at D2 or D5. Genes represented in this cluster were those involved in response to stimulus (adjusted $P < 0.05$) and the cytokine–cytokine receptor pathway categories (adjusted $P < 0.05$). Genes common to these two categories were *CCL20*, *CXCL1*, *CXCL6*, *IL-8*, *IL-18*, *IL-1β*, *TNFSF10*, *TNFSF13B* and *S100A8*.

Clusters 2 and 4 showed similar expression patterns, with both displaying increasing expression through to D5; however, the level of expression varied between replicate experiments in the two clusters. Genes represented in cluster 2 were involved in regulation of transcription and cellular

Table 3 Biological process (BP) and pathway (PW) ontologies ($P < 0.01$) of gene clusters from Fig. 1.

Cluster	Genes	Ontologies	*P*-value	Adjusted *P*-value
1	539	**Lipid metabolism (BP)**	2.0×10^{-7}	$\mathbf{5.7 \times 10^{-4}}$
		Sterol biosynthesis (BP)	2.4×10^{-7}	$\mathbf{3.5 \times 10^{-4}}$
		Sterol metabolism (BP)	2.1×10^{-5}	$\mathbf{1.2 \times 10^{-2}}$
		Alcohol metabolism (BP)	5.3×10^{-5}	$\mathbf{2.5 \times 10^{-2}}$
		Lipid biosynthesis (BP)	1.9×10^{-4}	6.6×10^{-2}
		Phospholipid metabolism (BP)	6.5×10^{-3}	8.2×10^{-1}
2	1016	**Regulation of transcription (BP)**	1.4×10^{-11}	$\mathbf{4.1 \times 10^{-8}}$
		Regulation of cellular metabolism (BP)	2.8×10^{-7}	$\mathbf{2.8 \times 10^{-7}}$
		Glutathione metabolism (PW)	5.7×10^{-3}	6.7×10^{-1}
		DNA packaging (BP)	7.6×10^{-3}	8.2×10^{-1}
		Chromatin modification (BP)	9.7×10^{-3}	8.7×10^{-1}
3	218	Amino acid biosynthesis (BP)	5.6×10^{-3}	9.6×10^{-1}
		Regulation of cell growth (BP)	6.2×10^{-3}	9.5×10^{-1}
		Cell proliferation (BP)	6.8×10^{-3}	9.4×10^{-1}
4	645	Regulation of transcription (BP)	2.7×10^{-4}	3.2×10^{-1}
		Regulation of cellular metabolism (BP)	3.0×10^{-4}	2.5×10^{-1}
		Immune response (BP)	5.1×10^{-4}	1.9×10^{-1}
		JAK-STAT cascade (BP)	6.8×10^{-3}	5.9×10^{-1}
5	275	**Response to stimulus (BP)**	4.5×10^{-6}	$\mathbf{2.6 \times 10^{-3}}$
		Cytokine–cytokine receptor interaction (PW)	1.3×10^{-4}	$\mathbf{2.5 \times 10^{-2}}$
		Proteasome (PW)	5.7×10^{-4}	5.3×10^{-2}
		Epithelial cell signalling in *Helicobacter pylori* infection (PW)	2.3×10^{-3}	1.4×10^{-1}
		Regulation of cell proliferation (BP)	5.2×10^{-3}	6.6×10^{-1}
		Toll-like receptor signalling pathway (PW)	6.4×10^{-3}	2.6×10^{-1}
		Cell–cell signalling (BP)	8.7×10^{-3}	7.8×10^{-1}
		Chemotaxis (BP)	9.1×10^{-3}	7.7×10^{-1}
6	495	ECM-receptor interaction (PW)	2.5×10^{-3}	3.5×10^{-1}
		Generator of precursor metabolites and energy (BP)	7.8×10^{-3}	1

Ontologies with a BH adjusted *P*-value <0.05 are shown in bold.

Table 4 Prolactin responsive genes ($P < 0.01$) identified from within the 3188 genes differentially expressed between D1 and D5.

Gene symbol and Affy ID	Gene name	Known functions	Response to prolactin	*P*-value
CSN1S1 Bt.3683.1.S1_at	Casein alpha S1	Milk protein	Up	**<0.0001**
SOCS2 Bt.13273.1.S1_at	Suppressor of cytokine signalling 2	Negative regulator of prolactin signalling, apoptosis	Up	**<0.0001**
CSN2 Bt.5381.1.S1_at	Casein beta	Milk protein	Up	0.0001
ELMO1 Bt.16985.1.S1_at	Engulfment and cell motility 1	Cell motility, apoptosis	Up	0.0019
SLC39A10 Bt.18309.1.A1_at	Solute carrier family 39, member 10	Zinc transporter, cell migration	Down	0.0020
LOC144404 Bt.21614.1.A1_at	Similar to TM protein induced by TNFalpha		Up	0.0025
SPOCK2 Bt.2520.1.S1_at	Sparc/osteonectin, cwcv & kazal-like domains proteoglycan (testican) 2	ECM component, cell migration	Down	0.0028
LITAF Bt.9286.1.S1_at	Lipopolysaccharide-induced TNF factor	Transcription factor, apoptosis	Up	0.0030
PPM1H Bt.12220.1.S1_at	Protein phosphatase 1H		Down	0.0050
LRRC35 Bt.15811.1.A1_at	Leucine rich repeat containing 35	Regulator of tubulin stability	Down	0.0050
COL4A6 Bt.19322.1.A1_at	Collagen, type IV, alpha 6,	ECM protein	Down	0.0077
FRS2 Bt.16219.1.A1_at	Fibroblast growth factor receptor substrate 2	Cell proliferation, motility and adhesion	Biphasic	0.0081
ZFP36L2 Bt.19706.1.S1_at	Zinc finger protein 36, C3H type-like 2	Probable role in regulating response to growth factors, apoptosis	Up	0.0086
Bt.24148.1.A1_at	No annotation		Down	0.0087
TPPP Bt.20088.1.S1_a_at	Tubulin polymerization promoting protein	Promotes microtubule stability	Down	0.0089
FLJ10178 Bt.24112.1.A1_at	Hypothetical protein	Nucleic acid binding	Down	0.0090
C5orf13 Bt.3780.1.S1_at	Chromosome 5 open reading frame 13	Cell migration, alveolar formation	Up	0.0091
BOC Bt.690.1.S1_at	Brother of CDO	Differentiation of skeletal muscle cells	Up	0.0091

Genes with a BH adjusted *P*-value <0.05 are shown in bold.

metabolism (both adjusted $P < 0.001$). Cluster 4 included genes involved in regulation of transcription, and regulation of cellular metabolism, as well as in the immune response and the JAK-STAT cascade (all unadjusted $P < 0.01$). *B4GALT1* (*β-1,4-galactosyltransferase*), which has a key role in lactose biosynthesis, was also present in cluster 4; however, *LALBA* (*α-lactalbumin*) was not differentially expressed and was expressed at extremely low levels. Cluster 3 showed highest expression levels on D5 and had similar expression profiles to clusters 2 and 4, albeit with higher levels of expression on D0 compared with clusters 2 and 4. Within this cluster were genes involved in amino acid biosynthesis and regulation of cell growth and proliferation (unadjusted $P < 0.01$).

Within the 3188 genes differentially expressed between D1 and D5, those that responded to Prl treatment on either D2 and/or D5 were identified. The 18 Prl-responsive genes ($P < 0.01$) are listed in Table 4. Only the top two genes, *CSN1S1* and *SOCS2*, had a BH-adjusted *P*-value <0.05, and the permutation-based method estimated the FDR of the 18 gene set at 22%. Nine genes were up-regulated in response to Prl and eight were down-regulated.

To validate results obtained from the microarray data, qRT-PCR analyses were performed on the four casein genes: *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3*, as well as *SOCS2*, *ELMO1* and *BOC*. Correlation analysis of the microarray vs. qRT-PCR data showed that there was good agreement between the two expression data sets (all $P < 0.0001$),

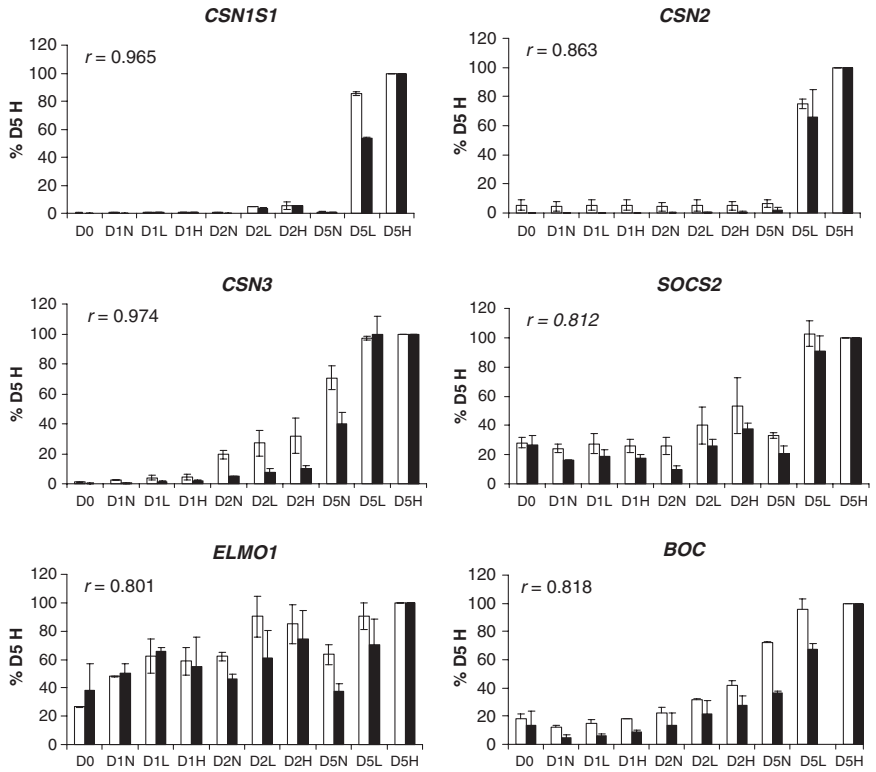

Figure 3 qRT-PCR confirmation of microarray expression profiles for selected genes. White bars, microarray data; black bars, qRT-PCR data. Results are presented as mean ± SEM. To enable direct comparison between the data, expression has been shown as a % of the expression level in the D5, high Prl treatments. D0, D1, D2 and D5 refer to the day of culture, N, L and H refer to the Prl concentration (none, low and high respectively). Correlations (*r*) between microarray and qRT-PCR expression data are shown in the graphs.

with the highest correlation between qRT-PCR and microarray data seen for *CSN3* (Fig. 3) and the lowest for *ELMO1* (Fig. 3).

Further statistical analysis of the qRT-PCR data was performed using a different statistical model to confirm the Prl responsiveness of selected genes. A linear model (allowing for variance heterogeneity) of qRT-PCR data confirmed most of the microarray results, with *CSN1S1*, *CSN2*, *SOCS2* and *ELMO1* showing a significant difference in expression between Prl treatments on D2 and/or D5 (all $P < 0.001$). *CSN3* ($P = 0.062$), however, did not show a significant response to Prl. *BOC* expression showed the same trends in the qRT-PCR data, although they were not statistically significant ($P = 0.077$). The expression of the casein genes increased during MEC differentiation (Table 5). *CSN3* (*κ-casein*) was the most highly expressed casein gene at each measured time-point and was also the least Prl-responsive. *CSN1S1* (*αS1-casein*) was the second most highly expressed casein gene and was Prl-responsive on both D2 and D5, showing reduced expression levels with decreasing concentrations of Prl. *CSN2* (*β-casein*) was the more lowly expressed and was Prl-responsive on D5.

Table 5 Casein mRNA expression levels in mammospheres, determined by qRT-PCR.

Treatment	Normalized mRNA expression		
Day [Prl]	*CSN1S1*	*CSN2*	*CSN3*
D0 N	0.00	0.00	0.25
D1 N	0.03	0.00	0.93
D1 L	0.28	0.01	2.65
D1 H	0.46	0.01	4.36
D2 N	0.09	0.02	9.61
D2 L	2.48	0.09	15.09
D2 H	3.93	0.16	20.30
D5 N	0.35	0.78	78.21
D5 L	38.88	32.96	197.83
D5 H	72.45	52.44	200.46

Values are expressed as (gene of interest copy number/housekeeping gene copy number) × 100.

Discussion

This study describes the characterization of an *in vitro* mammosphere model by microarray analysis. The primary BMEC cultured on basement membrane (Matrigel) formed alveoli-like mammospheres and behaved in a similar manner to previous reports (Barcellos-Hof *et al.* 1989; Rose *et al.* 2002). The initiation of alveoli-like morphology formation

in the present study was primarily driven by the ECM provided, as mammospheres formed even in the absence of Prl and essentially the same genes were differentially expressed during alveolar formation in the presence and absence of Prl, with only the level of expression changing. The presence of lactogenic hormones has been previously reported to be unnecessary for *in vitro* lobule formation (Rose *et al.* 2002) and Prl is not essential for lobuloalveolar development in murine mammary gland (Naylor *et al.* 2003). We considered the possibility that few Prl-responsive genes were detected because of the effects of endogenous Prl (Neville *et al.* 2002; Naylor *et al.* 2003). However, the microarray expression profile for Prl in the present study showed Prl expression was not above background in BMEC under these conditions (data not shown). The transition in gene expression that occurred after the first 24 h in culture, with many genes undergoing down-regulation and other genes starting to increase in expression, suggests that a distinct set of genes is involved in the initial mammosphere formation and that a separate set of genes may be required for milk production.

Expression of several ECM components on D1 of mammosphere development suggests that BMEC may contribute to formation of the surrounding basement membrane. Mammary basement membrane contains collagen IV and laminins, which are cross-linked by nidogens to form a gel-like structure to which MEC adhere (Katz & Streuli 2007). Secretion of collagen IIα1, collagen XIα2, γ1-laminin and γ2-laminin may supplement any species-specific deficiencies in the murine-derived basement membrane used in this model system. However, secretion of ECM components has been reported in mouse MEC cultures grown on Matrigel (Barcellos-Hof *et al.* 1989) and also in BMEC cultured on bovine derived ECM (Matitashvili & Bauman 2001), suggesting that MEC normally contribute to ECM formation. Integrins, which were highly expressed on D1, bind ECM and promote the formation of multi-protein complexes at the plasma membrane, which are focal centres for assembling the cytoskeleton and signalling platforms that control cell behaviour.

Expression of genes responsible for lipid and cholesterol biosynthesis is associated with the secretory activation phase at the onset of lactation and is necessary for milk fat production (Rudolph *et al.* 2003; Naylor *et al.* 2005b). Genes involved in lipid and sterol biosynthesis and metabolism were highly expressed in D1 mammospheres, suggesting that BMEC rapidly start the differentiation process once in contact with ECM.

It remains unclear whether lactose biosynthesis, a marker of MEC differentiation, was occurring in the mammosphere model. *LALBA* was poorly expressed, however, *B4GALT1* was up-regulated between D1 and D5. In mice, *α-lactalbumin* gene expression was up-regulated during lactation, while *β-1,4-galactosyltransefearse* expression was unchanged (Rudolph *et al.* 2007). Lactose synthesis has been detected in cultured bovine and murine alveoli (Cline *et al.* 1982; Baumrucker *et al.* 1988), however, there are no reports of lactose synthesis in mammospheres. Further investigation is required to establish whether the mammosphere model can synthesize lactose.

Milk protein expression requires gene activation of the JAK-STAT pathway, and maximal expression occurs in fully differentiated alveoli *in vivo*. Members of the JAK-STAT pathway were highly expressed on D5, including *STAT5a* and *STAT5b*, which encode the transcription factors that activate casein expression. Coincident with this was the expression of genes required for amino acid biosynthesis, presumably to supply the demand for milk protein expression. Regulators of transcription were also highly expressed in D5 mammospheres, and again this is probably related to the increased metabolic activity of the cells associated with milk production.

Interestingly, genes responding to stimuli and cytokine activity were highly expressed in D1 mammospheres. Several of the genes common to these ontology categories were chemokines (*IL-8*, *IL-18*, *CCL20*, *CXCL6* and *CXCL1*), which are generally associated with chemotaxis of leucocytes. BMEC can contribute to an immune response when challenged by bacterial infection (Strandberg *et al.* 2005), however, the role of chemokines during mammary gland development remains unknown. Other genes within these ontologies, *IL-1β*, *TNFSF13B*, *TNFSF10* and *S100A8*, have been reported to play a role in differentiation and/or apoptosis in other cell types and may also do so in BMEC where apoptosis is required for lumen formation (Debnath *et al.* 2002).

This study has revealed genes associated with *in vitro* mammosphere formation and highlights that key biological processes seen to be involved in this model are similar to those observed in other MEC culture systems. Other studies of MEC differentiation which were performed on tissue culture plastic in the presence and absence of lactogenic hormones, without providing an ECM (Desrivieres *et al.* 2003, 2007), identified differential expression of proteasomal proteins, cytoskeletal components, molecular chaperones, calcium binding proteins and components of RNA processing pathways; however, several key processes known to be involved in differentiation were absent, suggesting that provision of ECM provides a superior model for studying MEC differentiation.

Relatively few genes were differentially expressed in response to Prl when compared with the effect created by ECM. This may indicate that Prl is not a primary driver of alveoli formation and/or that some of the impact of regulation by Prl may be observed at a post-translational level. Alternatively, it may be an artefact of this culture model. Other cell culture studies have also found few Prl responsive genes (Beaton *et al.* 1997) or proteins (Ball *et al.* 1988). Mouse Prl receptor knockout studies (Ormandy *et al.* 2003; Naylor *et al.* 2005b) have yielded greater insight into the

role of Prl in mammary gland development and function. Among the list of 18 genes differentially expressed between Prl treatments ($P < 0.01$) in the present study, only the top three genes, *CSN1S1*, *SOCS2* and *CSN2*, were already known to be Prl responsive.

In the present study, while *CSN1S1* and *CSN2* expression was Prl responsive, with limited expression in the absence of Prl, *CSN3* expression was less responsive to Prl, with the expression levels observed in the absence of Prl not changing with the addition of the hormone. Although the caseins are all chromosomally co-located, differences in their respective promoter regions have been reported (Alexander *et al.* 1988; Rijnkels *et al.* 1995, 1997). Interestingly, on D5, *CSN3* was the most highly expressed casein followed by *CSN1S1* and *CSN2*; *CSN1S2* was not detected. This is in contrast to studies in bovine mammary tissue where approximately equal levels of each casein mRNA were present in lactating animals (Bevilacqua *et al.* 2006). A comparison of casein expression levels during mammosphere development with *in vivo* levels during a lactation cycle will be the subject of future work in our laboratory.

The *SOCS* gene family attenuates signalling *via* the JAK-STAT pathways. *SOCS2* expression was up-regulated in response to Prl in the present study, suggesting that this gene product may play a key role in negative feedback of Prl signalling in BMEC.

Engulfment and cell motility 1 (*ELMO1*) has not been previously reported as being Prl-responsive and the effect of its up-regulation in response to Prl in BMEC remains unknown. In glomerular epithelial cells, overexpression of *ELMO1* resulted in increased expression of fibronectin and integrin-linked kinase, and inhibition of cell adhesion (Shimakazi *et al.* 2006). The gene endocing BOC (brother of CDO) was up-regulated in response to Prl in the microarray analysis, and showed the same trend when analysed by qRT-PCR. BOC and CDO (cell adhesion molecule-related/down-regulated by oncogenesis) form complexes that act to promote differentiation of myoblasts (Kang *et al.* 2002) and may play a similar role in differentiation of BMEC.

The results show that mammosphere formation was largely driven by ECM; however, we also identified several novel Prl-responsive genes with potential roles in alveolar formation. The mammosphere model mimics some aspects of lactogenesis with the expression of milk protein and fat synthesis genes during differentiation. High gene expression levels of αS1-casein, β-casein and κ-casein in the mammosphere make it a suitable model for lactation studies on milk protein biosynthesis and the differential expression of caseins observed may provide opportunities to explore the regulation of casein expression in this model and conduct studies, which may not be feasible *in vivo*. Similarly, the expression of lipid synthesis genes in the mammosphere suggests that these may be suitable for studies on milk fat biosynthesis. However, there are some limitations to the model, as evidenced by the poor expression of *CSN1S2* and *LALBA*, and these should be considered in any application of the mammosphere model to lactation studies. The patterns of gene expression levels and pathways activated in mammospheres in these studies have helped in delineating the functionality and limitations for the use of this experimental model in furthering our understanding of the regulatory processes controlling milk protein biosynthesis.

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References

Alexander L., Stewart A., Mackinlay A., Kapelinkskaya T., Tkach T. & Gorodetsky S. (1988) Isolation and characterization of the bovine *κ-casein* gene. *European Journal of Biochemistry* **178,** 395–401.

Ball R.K., Friis R.R., Schoenenberger C.A., Doppler W. & Groner B. (1988) Prolactin regulation of *β-casein* gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line. *EMBO Journal* **7,** 2089–95.

Barcellos-Hof M., Aggeler J., Ram T. & Bissell M. (1989) Functional differentiation and alveolar morphogenesis of primary mammary cultures on reconstituted basement membrane. *Development* **105,** 223–35.

Baumrucker C.R., Deemer K.P., Walsh R., Riss T.L. & Akers R.M. (1988) Primary culture of bovine mammary acini on a collagen matrix. *Tissue and Cell* **20,** 541–54.

Beaton A., Wilkins R. & Wheeler T. (1997) Lactation-associated and prolactin-responsive changes in protein synthesis in mouse mammary cells. *Tissue and Cell* **29,** 509–16.

Bevilacqua C., Hebling J., Miranda G. & Martin P. (2006) Translational efficiency of casein transcripts in the mammary tissue of lactating ruminants. *Reproduction, Nutrition and Development* **5,** 567–78.

Cirfrian E., Guidry A., O'Brien C., Keys J.J. & Marquardt W. (1994) Bovine mammary teat and ductal epithelial cell cultures. *American Journal of Veterinary Research* **55,** 239–46.

Cline P.R., Zamora P. & Hosick H.L. (1982) Morphology and lactose synthesis in tissue culture of mammary alveoli isolated from lactating mice. *In Vitro* **18,** 694–702.

Debnath J., Mills K.R., Collins N.L., Reginato M.J., Muthuswamy S.K. & Brugge J.S. (2002) The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. *Cell* **111,** 29–40.

Desrivieres S., Prinz T., Laria N.C.P., Meyer M., Boehm G., Bauer U., Schafer J., Neumann T., Shemanko C. & Groner B. (2003) Comparative proteomic analysis of proliferating and functionally differentiated mammary epithelial cells. *Molecular & Cellular Proteomics* **2,** 1039–45.

Desrivieres S., Kuhn K., Muller J. *et al.* (2007) Comparison of the nuclear proteome of mammary epithelial cells at different stages of functional differentiation. *Proteomics* **7,** 2019–37.

Gautier L., Cope L., Bolstad B. & Irizarry R. (2004) Affy-analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics* **20,** 307–15.

Iziarry R., Hobbs B., Collin F., Beazer-Barclay Y., Antonellis K., Scherf U. & Speed T. (2003) Exploration, normalization and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4,** 249–64.

Kang J., Mulieri P., Hu Y., Taliana L. & Krauss R. (2002) BOC, an Ig superfamily member, associates with CDO to positively regulate myogenic differentiation. *EMBO Journal* **21,** 114–24.

Katz E. & Streuli C. (2007) The extracellular matrix as an adhesion checkpoint for mammary epithelial function. *The International Journal of Biochemistry & Cell Biology* **39,** 715–26.

Kleinman H. & Martin G. (2005) Matrigel: basement membrane matrix with biological activity. *Seminars in Cancer Biology* **15,** 378–86.

Matitashvili E. & Bauman D. (2001) Synthesis of extracellular matrix proteins in bovine mammary epithelial cells. *In Vitro Cellular & Developmental Biology* **37,** 629–32.

Naylor M., Lockefeer J., Horseman N. & Ormandy C. (2003) Prolactin regulates mammary epithelial cell proliferation via autocrine/paracrine mechanism. *Endocrine* **20,** 111–4.

Naylor M., Li N., Cheung J. *et al.* (2005a) Ablation of β1 integrin in mammary epithelium reveals a key role for integrin in glandular morphogenesis and differentiation. *Journal of Cell Biology* **171,** 717–28.

Naylor M., Oakes S., Gardiner-Garden M., Harris J., Blazek K., Ho T., Foo C., Wynick D., Walker A. & Ormandy C. (2005b) Transcriptional changes underlying the secretory activation phase of mammary gland development. *Molecular Endocrinology* **19,** 1868–83.

Neville M., McFadden T. & Forsyth I. (2002) Hormonal regulation of mammary differentiation and milk secretion. *Journal of Mammary Gland Biology and Neoplasia* **7,** 49–66.

Ormandy C.J., Naylor M., Harris J., Robertson F., Horseman N.D., Lindeman G.J., Visvader J. & Kelly P.A. (2003) Investigation of the transcriptional changes underlying functional defects in the mammary glands of prolactin receptor knockout mice. *Recent Progress in Hormone Research* **58,** 297–323.

Rijnkels M., Kooiman P., Krimpenfort P., de Boer H. & Pieper F. (1995) Expression analysis of the individual bovine *β-*, *αS2-* and *κ-casein* genes in transgenic mice. *Biochemical Journal* **311,** 929–37.

Rijnkels M., Kooiman P., de Boer H. & Pieper F. (1997) Organization of the bovine casein locus. *Mammalian Genome* **8,** 148–52.

Riley L., Williamson P., Wynn P. & Sheehy P. (2008) Lactoferrin decreases primary bovine mammary epithelial cell viability and casein expression. *Journal of Dairy Research* **75,** 135–41.

Rose M., Aso H., Yonekura S., Komatsu T., Hagino A., Ozutsumi K. & Obara Y. (2002) *In vitro* differentiation of a cloned bovine mammary epithelial cell. *Journal of Dairy Research* **69,** 345–55.

Rudolph M., McManaman J., Hunter L., Phang T. & Neville M. (2003) Functional development of the mammary gland: use of expression profiling and trajectory clustering to reveal changes in gene expression during pregnancy, lactation and involution. *Journal of Mammary Gland Biology and Neoplasia* **8,** 287–307.

Rudolph M., McManaman J., Phang T., Russell T., Kominsky D., Serkova N., Stein T., Anderson S. & Neville M. (2007) Metabolic regulation in the lactating mammary gland: a lipid synthesizing machine. *Physiological Genomics* **28,** 323–36.

Sheehy P., Della-Vedova J., Nicholas K. & Wynn P. (2004) Hormone-dependent milk protein gene expression in bovine mammary explants from biopsies at different stages of pregnancy. *Journal of Dairy Research* **71,** 135–40.

Shimakazi A., Tanaka Y., Shinosaki T., Ikeda M., Watada H., Hirose T., Kawamori R. & Maeda S. (2006) *ELMO1* increases expression of extracellular matrix proteins and inhibits cell adhesion to ECMs. *Kidney International* **70,** 1769–76.

Strandberg Y., Gray C., Vuocolo T., Donaldson L., Broadway M. & Tellam R. (2005) Lipopolysaccharide and lipoteichoic acid induce different innate immune responses in bovine mammary epithelial cells. *Cytokine* **31,** 72–86.

Supporting information

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Appendix S1 Notes on microarray analysis and comparison of microarray and qRT-PCR data.

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