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Acute involution in the tammar wallaby: Identification of genes and putative novel milk proteins implicated in mammary gland function

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ABSTRACT

Marsupials provide a suitable alternative model to studying mammary gland involution. They have evolved a different reproductive strategy from eutherians, giving birth to an altricial young and secreting milk that changes in composition during lactation. In this study, we used a marsupial-specific EST microarray to identify 47 up-regulated genes during mammary gland involution in the tammar wallaby (*Macropus eugenii*). These include the pro-apoptotic tumour necrosis factor receptor superfamily 21 (*TNFRSF21*) gene, whose expression in the mammary gland has not previously been reported. Genes encoding putative novel milk proteins which may protect the mammary gland from infection were also found to be up-regulated, such as amiloride binding protein 1 (*ABP1*), complement component 1QB (*C1QB*), complement component 4A (*C4A*) and colony stimulating factor 2 receptor β (*CSF2Rβ*). Our results show that the marsupial reproductive strategy was successfully exploited to identify genes and putative novel milk proteins implicated in mammary gland involution.

1. Introduction

Involution of the mammary gland may occur as either a gradual or acute process [1]. Gradual involution begins after peak lactation and refers to the process that takes place when lactation slowly reduces milk production as the young begins consuming solid food while continuing to suckle from the mother [1]. On the other hand, acute involution is an abrupt process, characterised by the rapid accumulation of milk in the mammary gland due to the cessation of milk clearance [1]. It is also associated with an inflammatory response [2–4], due to the increased likelihood of bacterial infection [5].

Acute involution, which will be investigated in this study, consists of a reversible first stage characterised by the appearance of apoptotic mammary epithelial cells in the lumen of secretory alveoli and an irreversible second stage characterised by tissue remodelling [6,7]. Studies in the mouse have shown that the transition between these two phases is irreversible after 2 days [7] and is regulated by *STAT3* (approved mouse gene symbol: *Stat3*) in the presence of *NF-κB* (approved mouse gene symbol: *Nfkb1*) [8] and nitric oxide [9]. The absence of *Stat3* in the mouse has been shown to delay the initiation of the second phase of involution [10] and extend the time period of the reversible phase by up to 4 days [11]. The inflammatory response during murine involution may also be attributed to the activation of *Stat3* and *Nfkb1*, as both of these molecules have been shown to regulate inflammatory signalling [8].

Previous analyses examining mammary gene expression during acute involution have focused on eutherian mammals such as humans, mice, sheep and cows. One such study is the cDNA microarray analysis undertaken by Singh et al. [12] on bovine mammary involution. This study showed a significant decrease in expression of a number of major milk protein genes, including α-casein, β-casein, κ-casein, α-lactalbumin and β-lactoglobulin. These results are in agreement with a number of other studies, as reviewed by Hurley [13], that also showed a down-regulation of the β-lactoglobulin [14–17], α-lactalbumin [15–18] and casein genes [16,17] during eutherian involution. In contrast, Singh et al. [12] also found that the expression of lactoferrin, as well as a number of other antimicrobial genes, was significantly increased during involution. This is consistent with the results of the Northern analysis undertaken by Schanbacher et al. [16] that similarly showed an increased expression of lactoferrin during bovine involution. A second microarray analysis, that investigated mammary gland involution in the mouse [19], found a number of genes encoding immunoglobulins to also be up-regulated. It is probable that the up-regulation of genes involved in immune responses during acute involution is due to the increased susceptibility to inflammation and infection [5], as the accumulation of milk and

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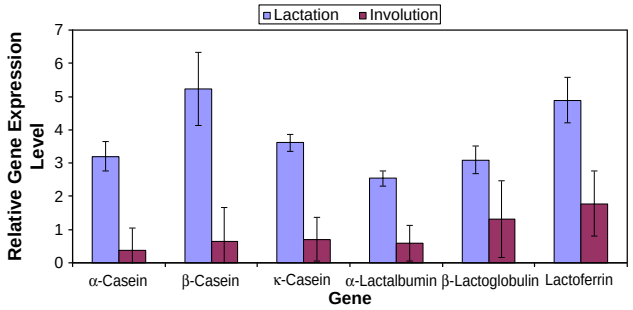

Fig. 1. Major milk proteins. Expression profiles of the differentially regulated major milk protein genes during tammar involution ($n=4$ for both lactation and involution samples).

slow clearance facilitates bacterial growth within the mammary gland and may also result in leakage from the teats with a subsequent enhanced route of infection.

The lactation strategy of the tammar wallaby (*Macropus eugenii*) is very different from eutherian species. The tammar exhibits a relatively short gestation, gives birth to an altricial young and has a long multi-phase lactation cycle characterised by major changes in the sucking pattern of the young and progressive changes in milk composition [20,21]. Phase 1 of lactation is comprised of a 26.5-day pregnancy followed by parturition. The subsequent 200 days of phase 2 are characterised by lactogenesis and the secretion of small volumes of dilute milk high in complex carbohydrate and low in fat and protein. The pouch young (PY) remains attached to the teat for approximately the first 100 days (phase 2A), after which it relinquishes permanent attachment to the teat and sucks less frequently while remaining in the pouch (phase 2B). The onset of phase 3 of lactation (200–330 days) is characterised by temporary exit from the pouch by the young, a large increase in milk production and a change in the composition of milk to include elevated levels of protein and lipid and low levels of carbohydrate [20,21].

Our study compared the changes in mammary gland gene expression in the tammar wallaby between lactating and involuting glands using microarray analysis. We used the results to identify the differentially regulated genes during acute involution in the tammar, particularly those that are up-regulated. Up-regulated genes encoding proteins with a signal peptide were identified as potentially new milk proteins that may have a role in regulating mammary gland involution and providing protection against infection.

2. Results

2.1. Differentially regulated genes

According to the criteria outlined in the experimental design, a total of 98 genes were found to be differentially expressed using microarray analysis. These include 47 up-regulated genes (Table 2) and 41 genes that were down-regulated (Table 3). The fold-changes and *P*-values of mean differences in expression between Lactation ($n=4$) and Involution ($n=4$) are presented in these tables. Up-regulated genes encoding proteins involved in mammary gland function during involution, namely protection against infection and apoptosis, are indicated in Table 2. The up-regulation of these genes, as well as genes that encode new putative milk proteins, as also indicated in Table 2, was validated using quantitative reverse transcription PCR (RT–PCR) (Fig. 4).

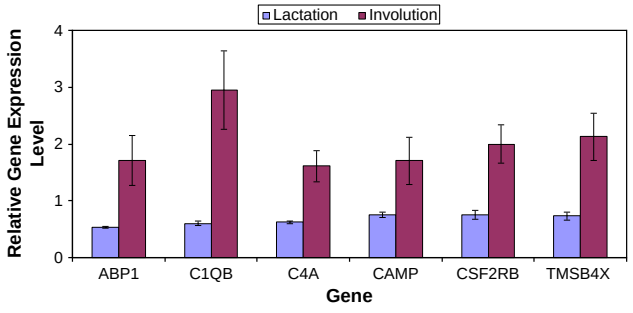

Fig. 2. Antimicrobial and immunostimulatory genes. Expression profiles of genes up-regulated during tammar involution that encode antimicrobial proteins and proteins involved in an immune response ($n=4$ for both lactation and involution samples).

2.2. Changes in expression of major milk protein genes

Expression of the α-casein (8.40-fold decrease; $P=0.0008$), β-casein (8.13-fold decrease; $P=0.0043$), κ-casein (5.13-fold decrease; $P=0.0001$), α-lactalbumin (4.31-fold decrease; $P=0.0001$), β-lactoglobulin (2.36-fold decrease; $P=0.0289$) and lactoferrin (2.75-fold decrease; $P=0.0056$) genes was down-regulated at involution (Fig. 1). The down-regulation of lactoferrin was confirmed by RT–PCR (Fig. 4). The transferrin, trichosurin and whey acidic protein (*WAP*) genes were not found to be differentially expressed during involution.

2.3. Up-regulated genes implicated in mammary gland function

The up-regulated genes on the array that are known to stimulate the immune system are *ABP1* (3.16-fold increase, $P=0.0409$) [22], *C1QB* (4.89-fold increase; $P=0.0144$), *C4A* (2.57-fold increase; $P=0.027$), *CSF2RB* (2.65-fold increase; $P=0.0027$) [23] and *TMSB4X* (2.92-fold increase; $P=0.0112$) [24] (Fig. 2). The only known milk antimicrobial gene on the array found to be up-regulated was the cathelicidin antimicrobial protein (*CAMP*) (2.27-fold increase; $P=0.0301$). The differentially expressed pro-apoptotic genes on the array during tammar involution are *SOCS3* (3.65-fold increase; $P=0.0231$) [25] and *TNFRSF21* (3.75-fold increase; $P=0.0229$) [26] (Fig. 3). The up-regulation of these eight genes was successfully validated using RT–PCR (Fig. 4).

2.4. Identification of new putative milk proteins encoded by up-regulated genes

The microarray analysis of mammary gland gene expression during tammar involution identified six up-regulated genes that were predicted to encode a signal peptide and whose expression has

Table 1
Primers for RT–PCR.

Primer	Sequence
ABP1 forward	5′ CCTATTCTTTCTGGCAACTATTTCTACT 3′
ABP1 reverse	5′ AGACTAGTAGTGCCATGATCACAATTC 3′
C1QB forward	5′ ATTGTTAGGTCTAACCTGAAGACAAAGT 3′
C1QB reverse	5′ AGAGAAACTTAGCAGGGAATAAACTGTA 3′
C4A forward	5′ GTTCTGCACTACACTCAAGATATCAAAG 3′
C4A reverse	5′ GTCTTCTCATAAGGTATCTCTTCAATCC 3′
CAMP forward	5′ ATCAGAGAGACTGTGTGCTCTAAAAGT 3′
CAMP reverse	5′ GGAGAGTTTTTCTGCTTCTCATGT 3′
CSF2RB forward	5′ AGATTATTTGATAGCCATCTCTCCACT 3′
CSF2RB reverse	5′ TTAAGCACTATCTATAAATGGCCCTATC 3′
GAPDH forward	5′ GACTGATGACTACAGTCCATGCCAT 3′
GAPDH reverse	5′ GTAAGCTTCCATGGAGAAATGGCCTCA 3′
Lactoferrin forward	5′ GTTAATTCTAGCCTCTGTGCCTTATGT 3′
Lactoferrin reverse	5′ CACAATAGTTCAAAGTCCTCACTCTTTA 3′
SOCS3 forward	5′ CATGTAGGAGATTCATGTGTTCTAAAAG 3′
SOCS3 reverse	5′ AGGCTAGAGAACTCCTGATTAGAGAAG 3′
TMSB4X forward	5′ GAAGAAGACAGAAACACAAGAGAAAAAC 3′
TMSB4X reverse	5′ CACAGTCATTTAAACTTTATCCAACCTC 3′
TNFRSF21 forward	5′ TTAATGTCAAGTCTCTTGTTCTCAACTC 3′
TNFRSF21 reverse	5′ CCATGTATACAAAGGATATAACAGGACA 3′

Table 2
Up-regulated genes during tammar involution.

Gene ID	Annotation	Fold-increase	*P*-value	Probes
ABP1[a,b]	Amiloride binding protein 1 (amine oxidase)	3.16	0.0409	1
ALDOA	Aldolase A, fructose-bisphosphate	3.28	0.0498	3
ANXA1	Annexin A1	3.82	0.0188	4
APLP2	Amyloid β (A4) precursor-like protein 2	3.43	0.0221	3
ARPC3	Actin related protein 2/3 complex, subunit 3, 21 kDa	2.82	0.0019	1
ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	2.98	0.0018	1
ATP6V1B1	ATPase, H^+ transporter, lysosomal 56/58 kDa, V1 subunit B1	2.70	0.0013	1
C15orf15	Chromosome 15 open reading frame 15	2.65	0.0116	1
C1orf93	Chromosome 1 open reading frame 93	4.89	0.0013	1
C1QB[a,b]	Complement component 1, q subcomponent, B chain	4.89	0.0144	1
C4A[a,b]	Complement component 4A (Rodgers blood group)	2.57	0.0270	5
CAMP[a]	Cathelicidin antimicrobial protein	2.27	0.0301	2
CCT6A	Chaperonin containing TCP1, subunit 6A (zeta 1)	2.80	0.0108	1
CD9	CD9 molecule	7.12	0.0008	1
CDR2L	Cerebellar degeneration-related protein 2-like	2.49	0.0184	1
CLU	Clusterin	12.24	0.0013	3
COL18A1	Collagen, type XVIII, α 1	2.71	0.0092	2
CSF2RB[a,b]	Colony stimulating factor 2 receptor, β, low-affinity	2.65	0.0027	1
CSRP1	Cysteine and glycine-rich protein 1	3.04	0.0472	1
CTSK	Cathepsin K (pycnodysostosis)	3.78	0.0076	1
CTSL	Cathepsin L	5.70	0.0491	3
EFS	Embryonal Fyn-associated substrate	2.50	0.0231	1
EIF2S3	Eukaryotic translation initiation factor 2, subunit 3 gamma	3.08	0.0011	1
FRAT1	Frequently rearranged in advanced T-cell lymphomas	4.25	0.0133	1
GLRX	Glutaredoxin (thioltransferase)	2.62	0.0031	2
GLUL	Glutamate-ammonia ligase (glutamine synthetase)	3.78	0.0331	2
GPX1	Glutathione peroxidase 1	5.80	0.0142	4
H3F3B	H3 histone, family 3B (H3.3B)	3.23	0.0075	1
HSP90AB1	Heat shock protein 90 kDa α (cytosolic), class B member 1	2.64	0.0152	1
ITGA9	Integrin, α9	2.64	0.0024	1
IVNS1ABP	Influenza virus NS1A binding protein	2.94	0.0031	1
KIAA1718	KIAA1718 protein	3.19	0.0120	2
LAMC3	Laminin, gamma 3	3.72	0.0319	1
LAPTM5	Lysosomal associated multispanning membrane protein 5	2.28	0.0480	1
LF	Lactoferrin	2.75	0.0056	1
NPM	Nucleophosmin (nucleolar phosphoprotein B23, numatrin)	3.76	0.0001	2
OXCT1	3-Oxoacid CoA transferase 1	2.85	0.0203	1
PSMA6	Proteasome (prosome, macropain) subunit, α type, 6	2.97	0.0038	1
PSMB8	Proteasome subunit, β type 8	2.43	0.0045	1
RPS3	Ribosomal protein S3	2.63	0.0097	1
SERPING1[b]	Serpin peptidase inhibitor, clade G, member 1	3.64	0.0350	2
SOCS3[a]	Suppressor of cytokine signalling 3	3.65	0.0231	1
SOD3[b]	Superoxide dismutase 3, extracellular	9.82	0.0478	2
TMSB4X[a]	Thymosin, β 4, X-linked	2.92	0.0112	1
TNFRSF21[a]	Tumour necrosis factor receptor superfamily, member 21	3.75	0.0229	1
TPT1	Tumour protein, translationally-controlled 1	2.78	0.0036	1
XPC	Xeroderma pigmentosum, complementation group C	3.22	0.0030	1

[a] Genes encoding proteins involved in mammary gland function during involution, namely protection against infection and promoting apoptosis.
[b] Genes that encode new putative milk proteins are underlined.

not previously been reported in the mammary gland. These genes are indicated in Table 2. In addition to the four proteins involved in immune responses (*ABP1*, *C1QB*, *C4A* and *CSF2RB*), other functions assigned to these six proteins include peptidase inhibition (*SERPING1*—3.64-fold increase; $P=0.035$) and anti-toxicity (*SOD3*—9.82-fold increase; $P=0.0478$) [27].

2.5. Comparison of gene expression during involution between mouse and tammar

In the microarray experiment conducted by Stein et al. [19] that analysed gene expression during mouse involution, 145 genes were found to be up-regulated, of which 49 were immunoglobulin genes. A further 16 genes were found to be down-regulated. None of the 49 up-regulated immunoglobulin genes appeared on the tammar array. This was due to the difficulty encountered in cloning antimicrobial genes into the pCMVSport 6.0 vector used to generate the cDNA tammar library used in the array. This vector was considered to be leaky to antimicrobials, justified by the small number of antimicrobial genes identified on the tammar array. Of the remaining 96 up-regulated genes, 25 were present on the tammar array, two of which were found to be up-regulated, *SOCS3* (3.65-fold, $P=0.0231$) and *CTSL* (5.70-fold increase, $P=0.0491$). Of the 16 down-regulated genes, 9 were present on the tammar array, one of which was found to be down-regulated, *G6PD* (2.74-fold decrease, $P=0.0087$).

2.6. Comparison of gene expression during involution between cow and tammar

A number of up-regulated genes in the microarray analysis conducted by Singh et al. [12], that analysed gene expression during bovine involution, were also found to be up-regulated during tammar involution. These genes include *ANXA1* (3.82-fold increase, $P=0.0188$), *CLU* (12.24-fold increase, $P=0.0013$), *GPX1* (5.80-fold increase, $P=0.0142$) and *TMSB4X* (2.92-fold increase, $P=0.0112$). It must also be noted that a few up-regulated genes in the tammar are very similar to genes found by Singh et al. [12] to be up-regulated in the cow. These include *CD9* (7.12-fold increase, $P=0.0008$; *CD6* and *CD24* in cow), *CTSK* (3.78-fold increase, $P=0.0076$; *CTSC* in cow), *CTSL* (5.70-fold increase, $P=0.0491$; *CTSC* in cow), and *SOD3* (9.82-fold

Table 3
Down-regulated genes during tammar involution.

Gene ID	Annotation	Fold-decrease	*P*-value	Probes
CETP	Cholesteryl ester transfer protein, plasma	5.86	0.0296	1
CSH1	Chorionic somatomammotropin hormone 1	5.25	0.0366	3
CSN1	α-Casein	8.40	0.0008	15
CSN2	β-Casein	8.13	0.0043	10
CSN3	κ-Casein	5.13	0.0001	1
CYP27A1	Cytochrome P450, family 27, subfamily A, polypeptide 1	2.88	0.0215	1
DERL2	der1-like domain family, member 2	2.91	0.0137	1
DGAT2	Diacylglycerol O-acyltransferase homolog 2 (mouse)	3.24	0.0205	3
DLC1	Deleted in liver cancer 1 transcribed locus	3.20	0.0099	1
FABP3	Fatty acid binding protein 3	6.15	0.0112	1
FBN1	Fibrillin 1	3.69	0.0032	1
G6PD	Glucose-6-phosphate dehydrogenase X-linked	2.74	0.0087	3
GALE	UDP-galactose-4-epimerase	2.38	0.0244	4
GSTT2	Glutathione S-transferase theta 2	4.24	0.0391	1
HFM	ATP-dependent DNA helicase homolog (S. cerevisiae)	4.81	0.0067	1
KIAA0310	KIAA0310 protein	5.28	0.0441	1
KIAA0513	KIAA0513 protein	3.31	0.0149	1
LALBA	α-Lactalbumin	4.31	0.0001	1
LGB	β-Lactoglobulin	2.36	0.0289	9
MCFD2	Multiple coagulation factor deficiency 2	3.16	0.0120	1
NEUROD6	Neurogenic differentiation 6	4.61	0.0232	1
NME1	Non-metastatic cells 1	3.83	0.0234	1
PCCB	Propionyl coenzyme A carboxylase, β polypeptide	3.41	0.0180	1
PRLR	Prolactin receptor	4.64	0.0116	1
QSCN6	Quiescin Q6	3.08	0.0298	1
RPS29	Ribosomal protein S29	5.24	0.0104	3
SEC11L3	SEC11-like 3 (S. cerevisiae)	2.70	0.0126	1
SIRT2	Sirtuin 2	4.96	0.0091	1
SLC28A3	Solute carrier family 28, member 3	3.00	0.0368	1
SLC36A1	Solute carrier family 36, member 1	3.12	0.0079	1
SLC5A6	Solute carrier family 5, member 6	5.37	0.0171	1
SLC6A14	Solute carrier family 6, member 14	4.98	0.0300	4
SND1	Staphylococcal nuclease domain containing 1	2.33	0.0293	1
TFRC	Transferrin receptor (p90, CD71)	3.14	0.0050	1
TM7SF2	Transmembrane 7 superfamily member 2	3.82	0.0057	1
TMEM142A	Transmembrane protein 142A	3.86	0.0009	1
TSPAN6	Tetraspanin 6	2.58	0.0270	1
YTHDC1	YTH domain containing 1	6.84	0.0035	1
ZNF700	Zinc finger protein 700	6.40	0.0131	1

increase, $P=0.0478$; *SOD2* in cow). It was additionally found by Singh et al. [12] that lipopolysaccharide binding protein (LBP) was up-regulated in bovine involution. This correlates with the up-regulation of *CAMP* (2.27-fold increase; $P=0.0301$) in the tammar, which is categorised as an LBP [28].

3. Discussion

The down-regulation of the α-casein, β-casein, κ-casein, α-lactalbumin and β-lactoglobulin genes observed in tammar involution is consistent with studies that analysed the expression of these genes during involution in other species [14–18]. Surprisingly, microarray analysis found lactoferrin gene expression to be down-regulated by 2.74-fold ($P=0.0056$) during tammar involution. RT–PCR also showed the down-regulation of this gene during involution. This result is unlike what was reported in studies of eutherian involution, where lactoferrin was found to be significantly up-regulated [16]. The concentration of lactoferrin in milk is elevated during phase 3 of tammar lactation [20], indicating that the gene may be expressed maximally at this time when the mammary gland is undergoing gradual involution. Since gradual involution in the tammar is much longer than in eutherians, the role of lactoferrin post-weaning may not be as important in the tammar and hence its expression characteristics during tammar involution.

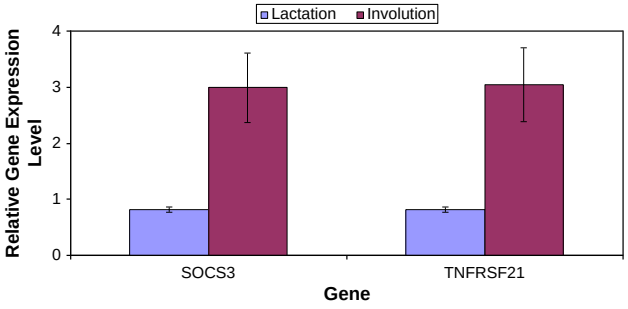

Fig. 3. Pro-apoptotic genes. Expression profiles of pro-apoptotic genes up-regulated during tammar involution ($n=4$ for both lactation and involution samples).

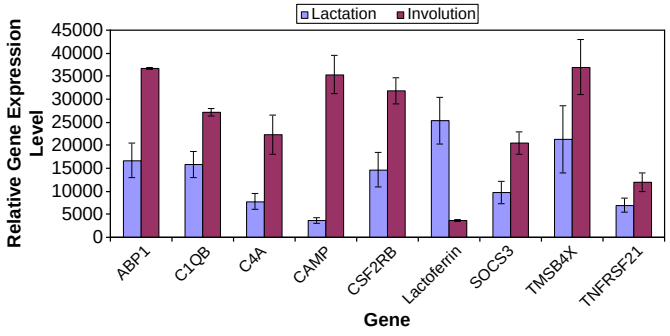

Fig. 4. RT–PCR expression profiles. RT–PCR validation of expression profiles of the up-regulated genes during tammar involution that encode immunostimulatory, antimicrobial and pro-apoptotic proteins ($n=3$ for lactation samples; $n=2$ for involution samples). Lactoferrin was included as a representative of the major milk proteins. Expression levels were calculated by normalisation against a tammar *GAPDH* housekeeper gene.

Transferrin, trichosurin and *WAP* genes were not found to be differentially expressed during tammar involution. The constant expression level of the *WAP* gene is consistent with the findings of a study in mice by Nakamura et al. [29], who showed that the expression of this gene in mice was unchanged 48 hours after weaning. *WAP* has been suggested to play an important role in mammary gland development [30] as well as tissue remodelling [31]. Although genes involved in mammary gland development are expected to be down-regulated during involution, the role of *WAP* in tissue remodelling, an event characteristic of involution, is likely to yield in a constant *WAP* expression level. It is also likely that transferrin, trichosurin and *WAP* are regulated by a different mechanism to the major milk protein genes that are significantly downregulated during involution, such as the casein, β-lactoglobulin and α-lactalbumin genes. Since milk is no longer secreted once the mammary gland has involuted, the continued expression of some milk protein genes at day 10 after the removal of the PY (RPY) suggests that these genes have not down-regulated completely within the time period analysed and may have another function yet to be identified.

Five genes on the array that affect various functions of the immune system were found to be up-regulated during tammar involution. *ABP1* is a diamine oxidase that catalyses the degradation of histamine [22], thereby decreasing the amount of swelling and inflammation. *CSF2RB* binds to cytokines [23] to stimulate immunity, while the complement components *C1QB* and *C4A* genes are involved in clearing pathogens. *TMSB4X,* which is also up-regulated in bovine involution [12], is involved in T-cell activation [24], plays an important role in killing pathogens and is known for its anti-inflammatory effects [32]. In addition to encoding a predicted leader sequence, the expression of four (*ABP1*, *C1QB*, *C4A* and *CSF2RB*) of these five genes has not previously been described in the mammary gland. Therefore, their encoded proteins are likely to be novel milk proteins that protect the mammary gland from potential infection during the period of milk stasis, which is associated with an increased risk of infection [5]. A further contributor in response to this potential infection is *CAMP*, also found in this study to be up-regulated during involution and whose encoded protein has been detected in the milk of mice and humans [33]. Given that *CAMP* is an LBP [28], this is consistent with the increase in expression of LBP in the cow [12].

Although expression of the *TNFRSF21* gene has not been reported in the mammary gland, its level of expression was significantly up-regulated during tammar involution. Also known as death receptor 6 (*DR6*), *TNFRSF21* encodes a receptor protein that functions in initiating activation-induced cell death [26] and is the only significantly up-regulated cell death receptor identified in the data set. The loss of *TNFRSF21* has also been found to cause an expansion of some leukocyte numbers [34], supporting its role in apoptosis. The up-regulation of *TNFRSF21* during involution is consistent with the study by Kasof et al. [35], who showed that *TNFRSF21* expression is induced by the activation of *NF-κB* through *TNFα* signalling. Although absent from the array, the *NF-κB* transcription factor is one of the most important regulators of apoptosis [36] and is rapidly induced following the onset of involution [37]. *TNFRSF21* can also stimulate *NF-κB* [38] to further propagate apoptosis.

The *SOCS3* gene, a signal transducer shown to promote apoptosis of mammary epithelial cells [25], has been observed to increase in expression by over 50-fold during day 1 of murine involution [25]. It has also been shown to strongly inhibit *STAT5* activation [39], which is required for the maintenance of lactation [40]. This is consistent with the down-regulation of *STAT5* by over 2.5-fold during tammar involution, although was not found to be statistically significant and hence does not appear in Table 3. Le Provost et al. [25] suggest that the inhibition of the *STAT5* gene during involution is due to the direct interaction between *SOCS3* and *PRLR*. This idea is supported by the results in this study, as expression of the *PRLR* gene was found to be down-regulated by 4.64-fold ($P = 0.0116$). Other studies of murine involution have shown that *SOCS3* (approved mouse gene symbol: *Socs3*) also modulates the expression of *Stat3* [41,42]. This correlates with the fact that *STAT3* is an important regulator of involution [10,11]. However, the expression profile of *STAT3* in the tammar was unable to be determined as the gene is absent from the array.

Besides *SOCS3*, the two other differentially expressed genes in tammar involution that were found by Stein et al. [19] to be differentially expressed in murine involution are *CTSL* and *G6PD*. *CTSL* is a protease whose up-regulation during involution is a likely result of the increase in protein concentration in milk at this time, reported in both tammar milk [43] and eutherian milk [44–46]. On the other hand, the down-regulation during involution of *G6PD*, which is involved in carbohydrate metabolism, is most likely due to the decrease in carbohydrate concentration in milk at this time, also reported in both tammar milk [43] and eutherian milk [44–46].

A number of up-regulated genes in bovine involution, as reported by Singh et al. [12], were also found in this study to be up-regulated in the tammar. *GPX1* and *SOD2* (*SOD3* in tammar) are of most interest due to their antioxidative properties, as antioxidative genes are known to be up-regulated during involution [12]. *SOD* proteins have been shown to protect cells from the toxic effects of highly reactive oxygen radicals intermediates by converting them to the less-reactive peroxide [27] that can then be destroyed by reactions with *GPX1* [47,48]. In addition to encoding a predicted leader sequence, the expression of *SOD3* has not previously been described in the mammary gland. Its encoded protein is likely to be secreted into the milk to protect the mammary gland against toxicity. Of further interest is the up-regulation of *CLU* in the tammar. In addition to its up-regulation in the cow [12], *CLU* is also up-regulated in murine involution [49]. Given the involvement of *CLU* in apoptosis [50], as well as its induction by reactive oxygen species, [51], the up-regulation of *CLU* in tammar involution strengthens the possibility that oxidative stress plays a role in apoptosis during mammary gland involution, as suggested by Singh et al. [12].

In addition to the genes encoding the *ABP1*, *C1QB*, *C4A, CSF2RB* and *SOD3* proteins, *SERPING1* was another up-regulated gene in tammar involution predicted to encode a novel milk protein. A peptidase inhibitor, *SERPING1*, may play a role in inhibiting enzymes such as those that break down peptides involved in the initiation of involution. The potential roles of these six proteins in mammary gland immunity and apoptosis during involution may involve their signalling of target cells through an autocrine mechanism which remains to be elucidated. This proposal is supported by Quarrie et al. [52] who found that the induction of apoptosis by sealing certain teats in lactating mice does not prevent the litter from suckling successfully on the remaining teats. Quarrie et al. [52] concluded that local control is a critical regulator of mammary gland cell death. In the tammar wallaby, a role of milk proteins in mammary gland function is also evident during asynchronous concurrent lactation, whereby the mother simultaneously provides milk for a PY lactating at phase 3, and milk of entirely different composition from an adjacent mammary gland for a newborn PY [20]. In addition to acting independently to produce milk of different composition, the two adjacent mammary glands involute at different times. This further supports the concept that mammary function is regulated by local factors, most likely by milk proteins that act through an autocrine mechanism [53].

4. Conclusion

This study adopted microarray analysis to identify a number of up-regulated genes that encode novel putative milk proteins secreted during tammar wallaby involution. These include *SOD3*, which is likely to protect against mammary gland toxicity, as well as *ABP1*, *C1QB*, *C4A* and *CSF2Rβ*, that are likely to protect the mammary gland from potential infection during the period of milk stasis. A further

contributor in response to this potential infection is *CAMP*, also found to be up-regulated. The genes identified to play a major role in the apoptotic cascade associated with marsupial involution are *SOCS3* and *TNFRSF21*.

5. Materials and methods

5.1. Tammars

We maintained the tammar wallabies (*M. eugenii*) in an open enclosure at The University of Melbourne Macropod Research Facility, Wantirna, Victoria, Australia, and provided feed and water *ad libitum*. Our care and treatment of the animals conformed to the National Health and Medical Research Council of Australia guidelines and were approved by The University of Melbourne Animal Experimentation Ethics Committee.

5.2. Microarray analysis of gene expression

We removed the PY from eight tammars at various times during mid-late lactation (days 168–260) and collected the adult mammary glands on either the day of RPY (Lactation; $n=4$) or 5–10 days following RPY (Involution; $n=4$). Microarray analysis, involving a tammar-specific array of 9888 genes expressed in the wallaby mammary gland, was undertaken by directly comparing the mean expression values of the lactation and involution samples using a dye-balanced design. The microarray experiments were undertaken as described by Brennan et al. [54]. Total RNA was firstly extracted using an RNeasy Lipid Tissue Mini-kit (Qiagen) following the manufacturer's instructions. RNA from each treatment group was labelled using amino-allyl reverse transcription followed by Cy_3 and Cy_5 coupling. Samples of total RNA (50 μg) were annealed at 70 °C for 10 min with 0.2 μg/ml T7 Anchored PolyT Primer (Geneworks), and reverse transcribed using amino-allyl dNTP mix (Sigma) and RNAse H enzyme for reverse transcription (Promega) at 42 °C for 2.5 h. We hydrolyzed the reaction mix by incubation at 65 °C for 15 min in the presence of 33 mM NaOH, 33 mM EDTA and 40 mM acetic acid. The cDNA was then adsorbed to a Qiagen QIAquick PCR Purification column. Coupling of either Cy_3 or Cy_5 dye was performed using incubation with adsorbed cDNA in 0.1 M sodium bicarbonate for 1 h at RT in darkness, followed by elution in 80 μl water. Labelled cDNA was further purified using a second Qiagen QIAquick PCR Purification column. Cy_3- and Cy_5-labelled probes in a final concentration of 400 μg/ml yeast tRNA, 1 mg/ml human Cot 1 DNA, 200 μg/ml polydT50, 1.2×Denharts, 1 mg/ml herring sperm DNA, 3.2×SSC, 50% formamide and 0.1% SDS were heated at 100 °C for 3 min. Probes were hybridised overnight at 42 °C in a humidified chamber and transferred to a custom tammar wallaby EST microarray, printed with 10000 ESTs from tammar mammary gland cDNA libraries generated from the tissue collected across the lactation cycle [55]. Microarrays slides were washed in 0.5×SSC, 0.01% SDS for 1 min, 0.5×SSC for 3 min then 0.006×SSC for 3 min at RT in the darkness. We scanned the slides with an Agilent Scanner and processed the resulting images using Biorad Versarray software to generate spot intensity data that were normalised using the Bioconductor software [56].

5.3. Data analysis

The statistical analysis was undertaken on the loess-normalised log ratios. In order to identify genes differentially expressed between lactation and involution, we used unpaired two-sided *t*-tests to compare the mean expression values between Lactation and Involution. Differential expression for all genes was considered using a significance threshold of $P<0.05$. Since only a small number of differentially expressed genes according to the microarray analysis were independently validated using quantitative RT–PCR, the major limitation associated with the analysis was the 'false discovery rate' (FDR). A significance threshold of $P<0.05$ resulted in an FDR of 16.5%, calculated using the *q*-value method described by Storey and Tibshirani [57]. To decrease this FDR, we eliminated all genes with a change in expression of <2-fold, hence also applying an element of theoretical significance to the results in addition to statistical significance.

5.4. Reverse transcription PCR

A total of five RNA samples used in the microarray analysis described above were also subjected to RT–PCR to confirm differential expression for a number of genes of interest. These RNA samples were extracted from tissue collected on either the day of RPY (Lactation; $n=3$) or 5–10 days following RPY (Involution; $n=2$). Reverse transcription of the DNAse-treated RNA was carried out at 42 °C for 1 h using SuperScript III reverse transcriptase (Invitrogen Life Technologies, Carisbad, CA) and 5 μg of total RNA. The reaction mix included 5 μl of 500 μg/ml oligo-dT (Promega, Sydney, Australia), 5 μl of 10 mM dNTPs (Invitrogen Life Technologies, Carisbad, CA), 20 μl of 5× FSB (Invitrogen Life Technologies, Carisbad, CA), 10 μl of 0.1 M DTT (Invitrogen Life Technologies, Carisbad, CA) and 200 U of RNasOUT (Invitrogen Life Technologies, Carisbad, CA). For PCR, 1 μg of the reverse transcription reaction was used as a template with 1 μl of each 10 μM primer (Table 1), 1 μl of 10× Optimised DyNAzyme EXT buffer (Finnzymes, Espoo, Finland), 1 μl of 10 mM dNTPs and 2 μl of 1 mM $MgCl_2$. Amplification was performed using 1 cycle of 94 °C for 5 min, followed by 33 cycles of 94 °C for 30 s, 60 °C for 30 s and 74 °C for 30 s and a final extension of 72 °C for 10 min. PCR products were electrophoresed in 1.5% agarose gels and viewed following ethidium bromide staining. Quantification was performed by densitometry using ImageJ software (NIH) with expression levels estimated by normalisation against a tammar *GAPDH* control (see Table 1 for primers).

5.5. Identification of genes encoding secreted proteins

Genes that were differentially expressed were assessed for the presence of a signal peptide. This was undertaken using the protein encoded by the human orthologues of the tammar sequences (BLAST score $<e^{-30}$), as the entire coding region for the tammar genes were often unavailable [55]. The signal peptides searches were carried out using the UniProt (http://www.ebi.uniprot.org), SignalP [58] and SPScan (GCG) [59] resource databases. There was a high correlation between the predictions of the three databases, as 92% of the proteins were predicted to be secreted by at least two of the methods used for analysis. Literature searches were subsequently undertaken on these putative milk proteins to determine whether they had previously been found to be expressed in the mammary gland.

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