Paracrine control of milk protein gene expression in tammar wallaby

by

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Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Deakin University

May, 2013
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Abstract

Due to its extreme lactation strategy the tammar wallaby (*Macropus eugenii*) provides unique opportunities to understand the local control of mammary epithelial cell function. The tammar lactation is characterised by a relatively short gestation period (phase 1) leading to the birth of a fetus–like young followed by a long lactation divided into three phases (2A, 2B and 3). At parturition only organ systems necessary for birth and immediate survival out of the uterus are functional. Quite intriguingly, despite its altricial state the neonate is able to locate one of the four available teats and attach for the first 100 days post-partum implying that the olfactory system is one of the earliest organ systems to develop. However the neonate is not capable of immune response and relies heavily on the mother’s capacity to progressively produce milk of profoundly different composition in each of the lactation phases in order to meet the specific immunological and nutritional needs at different stages of development. Although there is evidence to show that nursing tammars deliver immune factors through milk to the neonate, prior to the bactericidal activity assays reported in this study no antimicrobial activity had been demonstrated for any of the putative defence molecules. The current study examines how the level of cathelicidins, a family of antimicrobial proteins secreted in milk, progressively changes during lactation in relation to the potential microbial challenge of both the pouch young and the mammary gland of the mother.

Interestingly the lactating wallaby is capable of asynchronous concurrent lactation (ACL) in which the mother produces phase 2A milk from one mammary gland and phase 3 milk from an adjacent mammary gland concurrently in order to simultaneously feed the pouch young and an older sibling that has moved out of the pouch and started foraging. While phase 2A (P2A) milk is dilute and rich in oligosaccharides, phase 3 (P3) milk is concentrated and low in carbohydrates but rich in protein and lipid. Since the secretion of different milks from the
two mammary glands happens while the glands are under an identical systemic control, understanding the mechanisms regulating ACL has the potential to reveal factors within the mammary gland that control mammary epithelial function. The study investigates the role of the extracellular matrix (ECM) in the regulation of ACL and further examines the specific effect of ECM on the expression of individual milk protein genes with a focus on cathelicidin.

Using RTPCR we observed temporal regulation of cathelicidins through differential splicing synchronised with the need to protect the mother’s mammary gland and the neonate from possible infections. MaeuCath1 was found to be differentially spliced into two variants, MaeCath1a and Maeucath1b. MaeuCath1a consists of all the cathelicidin exons including the antimicrobial region and is highly expressed in early lactation and at involution, periods representing highest vulnerability of both the mother’s mammary gland and the neonate to pathogenic infections. MaeuCath1b has the antimicrobial region spliced out and is expressed predominantly in mid to late lactation when milk production and therefore mammary cell proliferation is at its peak. The study showed for the first time that MaeuCath1b has a proliferative effect on wallaby mammary epithelial cells (WallMECs). The study also showed that treatment of cells with mammary ECM from different phases of lactation reproduces the cathelicidin expression profile observed in mammary tissues from the respective phases. For instance treatment of WallMECs from P2A with ECM from P2B and P3 significantly decreased MaeuCath1a expression while treatment with P2A and involuting ECM increased its expression. Notably the study showed that treatment of WallMECs from an earlier phase of lactation with ECM from a later phase of lactation causes the cells to change their phenotype to the later phase.

Collectively this study has shown for the first time that lactation-phase specific changes in mammary ECM may be needed to direct ACL and the secretion of bioactives that regulate
mammary gland function and provide immunological and developmental signals to the pouch young.
Acknowledgements

With utmost sincerity I would like to express my gratitude to individuals and organisations who contributed to my studies in various ways and without whom the completion of this project would have not been possible. I would specifically want to thank the management of the Institute of Technology Research and Innovation (ITRI) for awarding me the scholarship and providing the laboratory space and equipment for my project.

I would like to sincerely acknowledge my principal supervisor, Professor Kevin Nicholas whose uncommon patience and deep insight in lactation biology made me feel at home with a lactation model whose uniqueness remains intriguing to many. As a mentor Kevin’s selfless commitment to the wholesome good of his students and ability to cultivate independent thinking daily energised me to attempt new goals without fear of failure. My heartfelt gratitude also goes to my co-supervisors, Assoc. Prof. Christophe Lefevre and Dr. Julie Sharp for patiently steering me through the rickety aspects of my project and the miry art of manuscript writing.

Throughout the duration of my studies I drew my motivation from knowing that my project would contribute to the understanding of the process of lactation and its ultimate benefits to human health. However, pending the outcome of my project, my life was greatly enriched by my participation in the K30000 Exercise- for- a- Cause, an initiative that raised thousands of dollars for medical clinics for the underprivileged in Kenya under the leadership of Pst. John and Anne Leach. To John and Anne, I would say thank you for leading by example and enriching my sense of purpose.

The pursuit of new goals always comes at the cost of normalcy and only true friends endure the goals of another. With utmost sincerity, I must acknowledge my wife Judy for sacrificing her normal work routine and taking on a strenuous work load to provide for the family during
the course of my study. To my children, Noah, Mercy, Levi and Gloria I am deeply grateful for inspiring me with your unswerving belief in me. You have been awesome.

Finally, I would like to acknowledge my parents William and Helmina Wanyonyi for being my unpaid life coaches and instilling in me transcendent virtues that have made relationships in and out of my workplace enjoyable. Mum and dad you deserve a pat on your shoulders.
Abbreviations

ACL  asynchronous concurrent lactation
BSA  bovine serum albumin
C/EBPα  CCAAT/enhancer-binding protein α
E₂  β17-estradiol
ECM  extracellular matrix
ELP  early lactation protein
EMSA  electrophoretic mobility shift assay
EST  expressed sequence tag
F  cortisol
GAPDH  glyceraldehyde-3-phosphate dehydrogenase.
GFP  green fluorescent protein
GR  glucocorticoid receptor
I  insulin
INV  involution
LLP-A  late lactation protein A
LLP-B  late lactation protein B
LPS  lipopolysaccharide
LTA  lipoteichoic acid
MMP  matrix metalloprotease
NF-1  nuclear factor 1
P  prolactin
P1  phase 1
P2A  phase 2A
P2B  phase 2B
<table>
<thead>
<tr>
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<tr>
<td>P3</td>
<td>phase 3</td>
</tr>
<tr>
<td>PY</td>
<td>pouch young</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>RNA-seq</td>
<td>RNA sequencing</td>
</tr>
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<td>RT-PCR</td>
<td>reverse transcription PCR</td>
</tr>
<tr>
<td>Sp1</td>
<td>specificity Protein 1</td>
</tr>
<tr>
<td>STAT5</td>
<td>signal transducer and activator of transcription 5A</td>
</tr>
<tr>
<td>T3</td>
<td>tri-iodothyronine</td>
</tr>
<tr>
<td>TGIF</td>
<td>TG-interacting factor</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitors of metalloprotease</td>
</tr>
<tr>
<td>WallMEC</td>
<td>wallaby mammary epithelial cells</td>
</tr>
<tr>
<td>WAP</td>
<td>whey acidic protein</td>
</tr>
<tr>
<td>YY1</td>
<td>yin yang 1</td>
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</table>
Table of contents

Access to thesis - A .............................................................................................................. i
Declaration .......................................................................................................................... ii
Abstract .............................................................................................................................. iii
Acknowledgements ......................................................................................................... vi
Abbreviations .................................................................................................................... viii
Table of Contents ............................................................................................................. x

Chapter One ....................................................................................................................... 1

Literature Review .............................................................................................................. 2
  1.0 Introduction .................................................................................................................... 2
  1.1 Reproduction and lactation in monotremes ................................................................. 2
  1.2 Reproduction and lactation in eutherians ................................................................. 3
  1.3 Reproduction and lactation in marsupials ............................................................... 5
  1.4 Lactation in wallaby .................................................................................................... 7
    1.4.1 Carbohydrates ..................................................................................................... 11
    1.4.2 Lipids .................................................................................................................. 12
    1.4.3 Protein – phase specific markers and their possible roles .................................. 12
  1.5 Phase specific markers of tammar lactation ............................................................ 13
    1.5.1 Phase 2A ............................................................................................................ 13
    1.5.2 Phase 2B .............................................................................................................. 14
    1.5.3 Phase 3 ............................................................................................................... 15
  1.6 Immunological molecules ......................................................................................... 16
  1.7 Anti-microbial and developmental proteins ........................................................... 18
    1.7.1 Cathelicidins ...................................................................................................... 18
  1.8 Hormonal control of lactation .................................................................................. 21
    1.8.1 Progesterone ..................................................................................................... 21
    1.8.2 Prolactin ............................................................................................................. 22
    1.8.3 Estradiol (E2) ..................................................................................................... 23
    1.8.4 Growth hormone ................................................................................................. 23
Chapter Four ..............................................................................................................................98

4.1 The Extracellular Matrix regulates MaeuCath1 expression ............................................. 99

4.1.1 Abstract .......................................................................................................................... 100
4.1.2 Introduction .................................................................................................................... 101
4.1.3 Results ........................................................................................................................... 103
4.1.4 Discussion ....................................................................................................................... 107
4.1.5 References ...................................................................................................................... 112
4.1.6 Materials and methods ................................................................................................. 115
4.1.7 Figures ............................................................................................................................ 120

Chapter Five ........................................................................................................................... 133

5.1 Transcriptome analysis (RNA-seq) of WallMEC cultured on ECM .............................. 133

5.1.1 Background ..................................................................................................................... 134
5.1.2 Methods .......................................................................................................................... 134
5.1.3 Results and discussion ................................................................................................. 137

Chapter Six .................................................................................................................................. 173

6.0 General discussion .............................................................................................................. 174

6.1 The wallaby as a lactation model ..................................................................................... 174

6.2 Key questions ..................................................................................................................... 174

6.2.1 MaeuCath expression and function during lactation ..................................................... 175

6.2.1.1 MaeuCath1a .............................................................................................................. 177

6.2.1.2 MaeuCath1b ............................................................................................................. 178

6.2.2 The role of the extracellular matrix (ECM) in wallaby lactation ................................ 180

6.2.3 ECM-regulated functional pathways in WallMEC ....................................................... 182

6.2.4 What regulates ECM composition? ............................................................................. 182

6.3 The role of ECM in MaeuCath1 gene expression ............................................................... 184

6.4 Possible methodological refinement .................................................................................. 185

6.5 Conclusion ......................................................................................................................... 186

7.0 Bibliography ........................................................................................................................ 187

8.0 Appendices .......................................................................................................................... 204
Chapter One
Literature Review

1.0 Introduction

The class Mammalia consists of an extremely diverse group of animals but they are all characterised by the production of milk to provide nutrition for the young. There are nearly 5000 species of mammals divided into three subclasses including the monotremes, marsupials and eutherians, (Sharman 1970, Tyndale-Biscoe, Hearn et al. 1974, Lefevre, Sharp et al. 2010). A distinctive feature of mammals is that although they occupy extremely diverse habitats, both terrestrial and marine, they maintain a constant body temperature thereby placing a significant demand on both the young and the adult to develop appropriate adaptive mechanisms which may include the consumption of high energy foods, hibernation (Terrien, Perret et al. 2011) and growth of hair among numerous others.

Notably, the diversity in the habitats also implies that each mammal is challenged by unique predatory or pathogenic challenges to which they have to adapt. For the mammalian neonate adaptation to the environment is packaged in the extremely divergent reproductive and lactation strategies observed across all the subclasses. Adaptive mechanisms that are common to all the three subclasses of mammals include the mother synchronising parturition with favourable environmental conditions and producing energy-rich milk (Conaway 1971). The following is a brief account of the various mammalian reproductive and lactation strategies:

1.1 Reproduction and lactation in monotremes

Monotremes belong to the order Monotremata and consist of 2 families, Ornithorhynchidae, the duck-billed platypus and Tachyglossidae which includes echidnas. A characteristic feature of the monotremes is that they are the only extant mammals in which the females lay eggs instead of giving birth. The egg is covered with a porous leathery shell which is capable of absorbing nutrients from the mother’s circulatory system (Behringer, Eakin et al. 2006)
during gestation and by the time it is laid it already has an embryo with well defined somites (Behringer, Eakin et al. 2006).

The female reproductive system and the eggs of monotremes closely resemble the reptilian and avian eggs but clear differences emerge in their gestation and incubation periods (Dawson 1983). Monotremes have longer gestation periods than extra-uterine incubations whereas birds and reptiles tend to have very short periods between development of the egg in the ovary and laying, but have extended periods of incubation. For instance while the platypus egg remains in the uterus for 28 days and hatches after ten days of extra-uterine incubation, in the chicken the egg is laid within 1 day of production in the ovary but has to incubate externally for 21 days before hatching (Dawson 1983).

There are some differences in the incubation and nursing habits among the monotremes. In the platypus, the egg is laid in a burrow on the side of the stream where the platypus lives and the young is hatched in an altricial state and is nursed by the mother which produces milk through pores in the abdominal skin (Holland and Jackson 2002). A major difference between the platypus and the echidna is that the latter has a pouch into which the egg is laid and incubated. Upon hatching, the young echidna remains in the pouch where it obtains milk from two areolae which are located on the anterior wall of the pouch (Holland and Jackson 2002).

1.2 Reproduction and lactation in eutherians

Unlike monotremes all eutherians are viviparous and in the majority of them the young is highly precocious when it is born, often requiring the mother’s nursing and protection only in the first few hours post-partum. A distinguishing feature of eutherian reproduction is that unlike monotremes and marsupials whose zygote derives nutrition from the yolk during the early phases of embryogenesis, the eutherian oocyte is very small, does not have a yolk and
therefore the developing embryo relies entirely on nutrition through the placenta. This is consistent with their classification as placental mammals (Rothchild 2003). Relative to marsupials and monotremes the eutherians give birth to well developed young. The eutherian reproductive cycle consists of hormonally-regulated development of the mammary gland, followed by pregnancy, varied lengths of gestation, parturition and lactation which varies in duration from species to species (Clutton-Brock and Harvey 1978). The duration of gestation in both eutherians and marsupials is thought to be dependent on body size of the mother and the degree of development of the neonate at parturition hence the larger mammals have relatively longer gestations than the smaller sized mammals (Huggett and Widdas 1951). Similarly the mammals that give birth to morphologically and physiologically well developed young have longer pregnancies than those that give birth to poorly developed young (Rowlands 1973, Lombardi 1998).

Exceptions to this rule include cetaceans, hystiricomorphs and the primates. Despite being among the largest mammals, cetaceans have a disproportionately short gestation - the blue whale (*Balaenoptera musculus*) is by far the largest extant mammal, weighing up to 180 metric tons and has a gestation that is only 1 to 3 months longer than that of the human (Lefevre, Sharp et al.). In contrast the hystiricomorphs which include the relatively diminutive guinea pigs and chinchilla rats give birth to precocious neonates after a relatively long gestation. The chinchilla for instance weighs only 500g but has a gestation of 111 days (Busso, Ponzio et al., Weir 1973, Kuroiwa and Imamichi 1977). Primates similarly have a higher gestation to weight ratio than expected but unlike hystiricomorphs, their young are less precocious and depend on maternal nursing for extended periods.

Whatever the species-specific mode of reproductive adaptation, eutherian neonates enjoy the benefit of longer gestations including predator evasion, reduced energy expenditure due to
transfer of the burden of thermo-regulation to the mother and a buffered physiochemical environment (Lombardi 1998).

Eutherian lactation is very diverse between species in regard to the timing of milk secretion (pre or post-partum), mammary gland morphology and frequency of suckling. Interspecies differences in milk composition are also evident (Medhammar, Wijesinha-Bettoni et al. 2011). At mid lactation for instance human milk consists of 0.8% protein compared to 10.6% in the domestic cat. Conversely carbohydrate content in human milk is 6.8% (Coppa, Gabrielli et al. 1993) compared to 3.7% in cat milk. However, the only major variation in the composition of eutherian milk occurs at the transition from the colostrum phase when the milk is concentrated (Saint, Smith et al. 1984) and rich in antibodies and antimicrobial proteins (Zablocka, Janusz et al. 2001), to the more dilute mature milk phase.

In humans mammary preparation for lactation starts at the onset of pregnancy but hormone-controlled development of milk ducts becomes evident 24 weeks after pregnancy. In the lead up to parturition the human mammary gland starts producing the immunoglobulin-rich colostrum which constitutes the first line of passive immunity conferred to the baby by the mother. However, before parturition production of mature milk is restricted by the presence of progesterone in circulation and is only initiated after birth when the level of progesterone declines, partly due to the ejection of the placenta (Conly, Morrison et al. 1970, Cregan, Mitoulas et al. 2002).

1.3 Reproduction and lactation in marsupials

The extreme divergence in mammalian reproduction and lactation is crystallised in the differences between marsupial and eutherian lactation. Compared to eutherians, marsupials have short gestation periods usually (lasting 26 days in tammar wallaby) (Tyndale-Biscoe, Hearn et al. 1974). The birth of the marsupial young happens briefly after gastrulation
allowing for limited pre-parturition organogenesis (Behringer, Eakin et al. 2006) and therefore only organs crucial for survival peripartum are developed when the young is born (Basden, Cooper et al. 1997, Old and Deane 2000). A major difference between eutherians and macropodid marsupials is that in the eutherians pregnancy must precede lactation whereas in marsupials the hormonal milieu during oestrus is sufficient to initiate lactation so long as a suckling stimulus is provided (Sharman and Galaby 1964, Findlay 1982). Additionally the four mammary glands of macropodid marsupials differentiate independently of each other and two adjacent mammary glands can produce milk of different composition simultaneously (Griffiths, McIntosh et al. 1972, Green, Newgrain et al. 1980, Findlay 1982, Nicholas 1988, Khalil, Digby et al. 2008).

Another distinguishing feature between marsupial and eutherian lactation is the timing of the mammary proliferation phase. In eutherians mammary proliferation ceases in the early post-partum period when peak lactation is attained and in some species even before parturition. For example, in mice, rats and rabbits cell proliferation continues upto 7 days post-parturition (Cowie, Forsyth et al. 1980). In contrast, in macropodid marsupials mammary growth (mediated by mitotic proliferation) appears to continue through the latter phases of lactation. However this sustained increase in the size of mammary gland has been a subject of debate (Tyndale-Biscoe 1987) as it was initially thought to be caused purely by the hypertrophic distension of mammary alveoli due to increased milk secretion (O'Donoghue 1911, Tyndale-Biscoe 1987). Later studies by Stewart, F. (Stewart 1984) however showed that mitotic cell division as well as hypertrophic distension contribute to the increase in mammary size during late lactation. Stewart measured DNA concentration in tammar wallaby mammary glands at days 140 and 240 lactation and found that although the volume of the mammary gland at day 240 was much higher than day 140, the DNA concentration was unchanged implying that there was a marked increase in cell numbers during peak lactation.
Due to their relatively short gestation, marsupial neonates are deprived of the advantages of long embryo retention including the transfer of the cost of thermo-regulation to the mother and the benefits of the pathogen-sequestered environment of the placenta, both of which are enjoyed by eutherian neonates (Lombardi 1998). Consequently the marsupials make a significantly higher investment than eutherians in postnatal development during lactation. Not surprisingly, while the composition of eutherian milk remains constant after the colostrum phase (Schmidt 1971, Jenness 1974), marsupials progressively alter the milk composition throughout lactation in order to meet the developmental and nutritional needs of the young (Green, Newgrain et al. 1980, Nicholas, Hartmann et al. 1981, Nicholas 1988, Nicholas, Fisher et al. 2001, Trott, Simpson et al. 2003, Lefevre, Digby et al. 2007, Khalil, Digby et al. 2008, Kwek, Iongh et al. 2009).

1.4 Lactation in wallaby

Tammar lactation is divided into four phases (figure 1). The gestation phase (P1) lasts approximately 26 days culminating in the birth of a fetus-like young (Renfree 1981, Tyndale-Biscoe 1987, van Oorschot and Cooper 1988). During P1 all the four mammary glands undergo progressive lobulo-alveolar development, gradually replacing the connective tissue with glandular tissue (Findlay 1982). Phase 2A (P 2A) starts at parturition when the neonate, although extremely altricial, has motile capability and crawls from the cloaca to one of the four teats to which it attaches permanently for approximately 100 days post-partum (Tyndale-Biscoe 1987, Nicholas 1988, Old and Deane 2000, Behringer, Eakin et al. 2006, Brennan, Sharp et al. 2007). Once the pouch young starts sucking, the other three mammary glands regress and become quiescent (Daly, Digby et al. 2007). During P2A the mother produces relatively small volumes of dilute milk that is rich in carbohydrates but low in protein and lipid (Nicholas 1988, Hendry, Simpson et al. 1998, Trott, Simpson et al. 2003) (figure 1). A notable feature of neonatal development during P2A is that the growth and development of
the brain occurs at a remarkably high rate compared to other organs (Renfree, Holt et al. 1982).

Phase 2B (P2B) commences 100-120 days post-partum and continues for approximately 100 days during which the neonate remains in the pouch but relinquishes the teat and only re-attaches to suck (Hendry, Simpson et al. 1998, Trott, Simpson et al. 2003). Similar to P2A the milk produced during P2B is dilute and high in carbohydrates but low in protein and lipids. Unlike P2A however, neonatal brain growth slows down during P2B (Renfree, Holt et al. 1982) and instead the development of organ systems required for survival outside the pouch is accelerated. For instance at around day 140 post-partum the eyes open thereby enabling vision, by day 160 the PY can stand on its own and by day 180 the thyroid gland is functional and therefore neonatal thermoregulation is possible (Renfree, Holt et al. 1982).

At the onset of phase 3 (P3), the neonate ventures out of the pouch and feeds on herbage, only returning to the pouch infrequently to suckle. During P3 the mammary gland enlarges significantly (Bird, Hendry et al. 1994), producing large amounts of concentrated milk that is rich in protein and lipid but low in carbohydrates (Tyndale-Biscoe 1987, Nicholas, Simpson et al. 1997, Hendry, Simpson et al. 1998, Trott, Simpson et al. 2003). P3 also represents the period of most dramatic change in morphology and growth of the young including the switch from ectothermic to endothermic regulation of body temperature (Nicholas 1988, Nicholas,
Figure 1.

Progressive changes in milk production, milk composition and growth of the young during the three phases of the lactation cycle in the tammar wallaby (Nicholas, Simpson et al. 1997, Brennan, Sharp et al. 2007).
Table 1: Comparison of milk protein, fat and carbohydrate content in mammals

<table>
<thead>
<tr>
<th></th>
<th>Protein (g)</th>
<th>Fat (g)</th>
<th>Carbohydrate (g)</th>
<th>Energy (kcal)</th>
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<td>3.7&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Tammar wallaby (P2A)</td>
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<td>238&lt;sup&gt;c&lt;/sup&gt;</td>
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</table>


1.4.1 Carbohydrates

Similar to eutherian milk (Kunz, Rudloff et al. 2000), the main carbohydrate in tammar milk at parturition is lactose (Green, Newgrain et al. 1980). However at around day 6 postpartum and for the rest of P2A and P2B lactose becomes less prominent and is replaced by complex oligosaccharides in the form of galactosyllactoses, [Gal(β-3)]nGal(β-4)Glu, which at this stage of lactation constitute as high as 50% of the total solids in tammar milk (Green, Newgrain et al. 1980, Nicholas 1988, Menzies and Nicholas 2007). The large increase in oligosaccharides is attributable to the increased expression of the genes for the anabolic enzymes such as β-1,3 galactosyltransferase (3βGalT) and β-1,4 galactosyltransferase (4βGalT) which catalyse the transfer of galactose to lactose (Messer and Green 1979, Messer and Nicholas 1991, Menzies and Nicholas 2007). It has been suggested that prior to day 6 postpartum the gut of the PY may not have started secreting the enzymes needed to digest the oligosaccharides hence the mother secretes milk that is rich in lactose instead of oligosaccharides (Messer and Green 1979, Messer and Nicholas 1991). Presumably once the neonate’s gastro-intestinal system acquires the ability to metabolise the complex sugars, the mother starts secreting milk that is high in oligosaccharides thereby delivering high energy milk to the PY without increasing its osmolarity (Messer and Elliott 1987).

Marking the transition from phase 2B to P3 there is a drastic decrease in total hexose in tammar milk (Messer and Green 1979, Green, Griffiths et al. 1983, Nicholas 1988). Additionally the level of complex oligosaccharides decreases significantly, coincident with increased activity of UDP-galactose hydrolase (Messer and Nicholas 1991) and instead the major carbohydrates are the monosaccharides glucose and galactose (Messer and Green 1979, Green, Griffiths et al. 1983). By this time the neonate will have acquired an adult-like kidney function and is able to metabolise concentrated milk of high osmolarity.
1.4.2 Lipids

The lipid content in tammar milk is represented mainly by triacylglycerols (Green, Griffiths et al. 1983, Kwek, Wijesundera et al. 2007) but the fatty acyl content is temporally regulated. During P2A palmitic acid (C\textsubscript{16:0}) is the predominant fatty acid in milk triacylglycerols but its level decreases by day 70 of lactation coincident with an increase in oleic acid (C\textsubscript{18:1}). Observations from an \textit{in-vitro} model using mammary explants from days 20-24 pregnant wallabies showed that although mammary epithelial cells can synthesize lipid when media is supplemented with insulin alone, secretion of lipid into the lumen can only happen when prolactin is included (Kwek, Wijesundera et al. 2007) suggesting that lipid content in milk is under hormonal control. Further, in this explant model, in the absence of prolactin the cells secreted more oleic acid than palmitic acid but on addition of prolactin, palmitic acid was the predominant fatty acid. Since palmitic acid is a constituent fatty acid of a major surfactant (R.V. Baudinette 1988) in the lung, its synthesis in P2A may suggest that it is required in the development of the respiratory system. Similarly, oleic acid, a precursor of nervonic acid which is required for the formation of myelin (Sargent, Coupland et al. 1994) may be crucial in brain development of the neonate (Kwek, Wijesundera et al. 2007).

1.4.3 Protein – phase specific markers and their possible roles

All the four major milk proteins β-lactoglobulin, α-lactalbumin, α-casein and β-casein are present in tammar milk at parturition (Trott, Simpson et al. 2003, Joss, Molloy et al. 2009) and remain throughout lactation. However at the onset of P3 the expression of the β-lactoglobulin and β-casein genes increases significantly while the expression of α-lactalbumin and α-casein genes remains unaffected (Bird, Hendry et al. 1994). A noteworthy feature of P2A is that at parturition the activity of beta-1,3 galactosyltransferase (\(3\beta\text{GalT}\)) an enzyme that catalyses the formation of oligosaccharides is undetectable but its activity increases six-fold by day 10 post-partum and its activity is dependent on the sucking stimulus.
of the neonate. A similar enzyme beta-1,4 galactosyltransferase is detectable on d1L but its activity also increases 3-fold by day 10 post-partum (Menzies and Nicholas 2007). Since the expression of 3βGalT has been shown to be inducible by lactogenic hormones in mammary explants extracted from pregnant wallabies, the increase in milk oligosaccharides is likely to be regulated by hormones and factors intrinsic to the mammary gland (Menzies and Nicholas 2007). The switch to P3 signals a decline in 3βGalT and up-regulation of UDP-galactose hydrolase which hydrolyses UDP-galactose thereby inhibiting the formation of oligosaccharides (Messer and Nicholas 1991).

1.5 Phase specific markers of tammar lactation

1.5.1 Phase 2A

In addition to the differential expression of the enzymes involved in oligosaccharide metabolism, each of the three post-parturition phases of tammar lactation is characterised by the expression of phase-specific proteins (Nicholas 1988, Nicholas, Simpson et al. 1997, Trott, Wilson et al. 2002, Trott, Simpson et al. 2003, Menzies and Nicholas 2007) (figure 2). P2A is characterised by the expression of the gene for the early lactation protein (tELP) (Simpson, Shaw et al. 1998, Trott, Simpson et al. 2003, Khalil, Digby et al. 2008), P2B by the expression the gene for the whey acidic protein (tWAP) (Simpson, Ranganathan et al. 2000, Nicholas, Fisher et al. 2001) and P3 by the expression of the gene for the Late lactation protein (LLP-B) (Trott, Wilson et al. 2002, Trott, Simpson et al. 2003).

The 18kDa tELP is a member of the Kunitz family of protease inhibitors and shares 37% homology with the bovine colostrum trypsin inhibitor (Simpson, Shaw et al. 1998, Pharo, De Leo et al. 2012). The expression of tELP stops when the PY relinquishes the teat for the first time and switches from being permanently attached to the teat to intermittent re-attachment for feeding (Simpson, Shaw et al. 1998). Although the physiological role of tELP still remains unclear, it has been suggested that its expression in early lactation is a safeguard
against proteolytic degradation of immunoglobulins (Ig) in the milk (Simpson, Shaw et al. 1998). A relationship indeed exists between both bovine (Pineiro, Brock et al. 1978) and porcine (Jensen and Pedersen 1979) colostrum trypsin inhibitor and the Ig content of milk suggesting that tELP may play an immunological role when the PY is most vulnerable to infection.

1.5.2 Phase 2B

Once the PY relinquishes the teat the expression of the gene for the whey acidic protein (tWAP), a member of the four-disulphide core (4-DSC) domain family of proteins commences and remains up-regulated until the PY vacates the pouch (figure 2). The gene for tWAP consists of three 4-DSC’s instead of two 4-DSC’s found in eutherians (Nicholas, Simpson et al. 1997, Simpson, Ranganathan et al. 2000, Demmer, Stasiuk et al. 2001, Nicholas, Fisher et al. 2001). Similar to tELP, tWAP is a putative protease inhibitor but its predicted active site has no similarity with the active sites of known protease inhibitors (Simpson, Ranganathan et al. 2000).

Since tWAP is a multi-domain protein it is feasible that it plays various roles in lactation and a three dimensional model of tWAP has indeed shown that its three 4-DSC’s have no molecular interaction with each other and are likely to act autonomously (Simpson, Ranganathan et al. 2000). Since the expression of the tWAP gene is at its peak when the PY starts developing fur and coincides with an increase in the milk content of sulphur-rich amino acids (Renfree, Meier et al. 1981, Simpson, Ranganathan et al. 2000) which have been associated with hair growth, it has been suggested that tWAP may be necessary for hair growth. Cell proliferation assays performed by measuring DNA synthesis and the expression level of cell-cycle regulators cyclin-D1 and CDK4 in mouse mammary cells (HC11) and wallaby mammary epithelial cells (WallMECs) have shown that domain III (DIII) of tWAP
enhances cell proliferation (Topcic, Auguste et al. 2009). These findings are particularly significant because peak tWAP secretion in milk occurs between d172L and d202L (Simpson, Ranganathan et al. 2000) when the mammary starts enlarging in the lead up to the exit of the neonate from the pouch and its subsequent consumption of large volumes of milk (Nicholas, Simpson et al. 1997). More recently, domain II of WFDC2, another 4-DSC family member was found to have antimicrobial activity against Staphylococcus aureus, a known agent of mastitis but no such activity against Enterococcus faecalis, a commensal of the gut, leading to the suggestion that it provides antimicrobial protection to the mother’s mammary gland while preserving the balance of the gut microflora (Watt, Sharp et al. 2012).

1.5.3 Phase 3

Overlapping with the expression of tWAP (figure 2) from around 26 weeks post partum is another developmentally regulated protein, the late lactation protein-A (LLP-A) which bears similarity to the lipocalin family of proteins (Trott, Wilson et al. 2002). Although it is well established that protein content, especially whey proteins increases dramatically at the onset of P3 (Green, Newgrain et al. 1980) it is intriguing that close to half of that increase is contributed by LLP-A which reaches a peak concentration of 26g/l between 36 and 40 weeks post partum (Nicholas, Messer et al. 1987). The timing of the secretion of LLP-A in milk with the shift from a purely milk diet to a combination of milk and herbage by the PY its relatively high concentration in milk suggests that LLP-A could play an anti-toxin role or a simple dietary role.

LLP-A shares 48% amino acid homology with another lipocalin-like protein, the late lactation protein (LLP-B) whose secretion in milk is detectable from d200L and remains until weaning (Trott, Wilson et al. 2002). Both LLP-A and LLP-B have been shown to be regulated by the hormones cortisol, insulin and prolactin (Trott, Wilson et al. 2002, Trott,...
Adams et al. 2005) and can only be expressed in mature alveoli (Trott, Wilson et al. 2002). Given their prominence in marsupial milk (Nicholas, Messer et al. 1987, Trott, Wilson et al. 2002, Trott, Simpson et al. 2003) and absence in eutherian milk these proteins are likely to play roles other than nutrition that are unique to marsupial lactation and therefore a need exists to investigate their role in neonatal development.

**Figure 2.**

Differential expression of the major milk proteins during tammar lactation *(Nicholas, K.)*
1.6 Immunological molecules

At parturition tammar PY have no functional immune system and only acquire adult-like immune mechanisms in late P2B when they are about to exit the pouch (Deane 1988, Basden, Cooper et al. 1997, Baker, Belov et al. 2005). The PY is therefore dependent on the delivery of immune factors through the milk for protection against pathogens which exist in the pouch (Old and Deane 1998) and the wallaby habitat. Accordingly, two periods of immune transfer from the mother to the neonate characterised by a dramatic increase in the expression of immunoglobulin genes in the mammary gland have been observed in tammar (Daly, Digby et al. 2007). The first period of increased expression of Ig’s occurs a few hours after parturition while the second one occurs just prior to the first exit of the pouch by the neonate, the two periods of greatest vulnerability to pathogens by the PY. Importantly, not only are all the major Ig’s including IgA, IgG, IgM, IgE, Igκ and Igλ upregulated but also the chemokine CCL28 which facilitates epithelial uptake of IgA (Wilson and Butcher 2004) and the IgG receptor FcRn (Adamski, King et al. 2000, Western, Eckery et al. 2003) Similar periods have been reported in another marsupial, the brushtail possum (*Trichsurus vulpecula*) (Adamski and Demmer 1999, Adamski and Demmer 2000, Adamski, King et al. 2000).

Notably, although immune transfers are well known in eutherians, they only happen in the colostrum phase after which the gastrointestinal epithelium closes and becomes impervious to Ig’s (Morris 1964, Kolb 2002). In contrast marsupial neonates including the tammar PY are capable of immunoglobulin absorption across the gastro-intestinal barrier throughout lactation (Yadav 1971, Daly, Digby et al. 2007). The tammar lactation model therefore presents unique opportunities to understand the role of passive immunity in neonatal development.
1.7 Anti-microbial and developmental proteins

Using a complex set of proteomics tools including the matrix assisted laser desorption ionisation mass spectrometry (MALDI MS) and de novo peptide sequencing, a mix of proteins associated with various functions including antimicrobial defence, growth and development have been identified in milk (Joss, Molloy et al. 2009). The study by Joss et al identified lactoferrin, complement B protein, haptocorrin, amphiphysin and the myeloid cathelicidin among other proteins, all of which were developmentally regulated. Lactoferrin and cathelicidin are well known antimicrobial proteins while haptocorrin has also been shown to have bacteriocidal activity against *E.coli* (Adkins and Lonnerdal 2003) and all of them were expressed in P2A and P2B (Joss, Molloy et al. 2009). Amphiphysin which plays a role in endocytosis (Butler, David et al. 1997) was found in d172L – d250L milk while complement B protein which plays a role in pathogen opsonisation (Peters, Ambrus et al. 1988) was detected in P2B.

1.7.1 Cathelicidins

Cathelicidins are a well studied family of antimicrobial proteins found in a wide variety of vertebrates (Zanetti, Gennaro et al. 1995, Boman 1998, Hancock and Lehrer 1998, Lehrer and Ganz 1999, Zasloff 2002). Together with many other cationic amphiphilic peptides collectively known as host-defense peptides (Hancock, Brown et al. 2006, Nijnik and Hancock 2009), cathelicidins represent a significant constituent of innate immunity in mammals and display a remarkably broad spectrum anti-pathogenic activity, a single cathelicidin often showing activity against bacteria, fungi and viruses (Ramanathan, Davis et al. 2002).

The generic primary structure of cathelicidins includes four exons (Gudmundsson, Magnusson et al. 1995, Zhao, Ganz et al. 1995, Gudmundsson and Agerberth 1999), the first
three of which consist of the 22-35 amino acid N-terminal signal peptide and the cathelin-like region which covers 90-115 amino acids (Yang, Biragyn et al. 2004). The fourth exon consists of the variable C-terminal anti-microbial domain preceded at its N-terminal by a species-specific protease cleavage site (Zanetti, Litteri et al. 1991, Gudmundsson, Agerberth et al. 1996). Due to the variability in the amino acid composition of the antimicrobial region of cathelicidins their structural conformation also varies between species, often existing in α-helical (Hirata, Shimomura et al. 1994), extended α-helical (Cabiaux, Agerberth et al. 1994) and β-sheet conformations (Fahrner, Dieckmann et al. 1996). All cathelicidins however share a net positive charge of 2-9 and a high percentage of hydrophobic residues. This mix of cationic and hydrophobic residues confers amphipathic properties to cathelicidins upon which their antimicrobial activity is dependent (Hancock and Lehrer 1998, Zasloff 2002). Although the mechanism by which cathelicidins kill their targets is not very well understood, its consensual that amphipathic interactions with pathogen membranes play a major role (Hancock and Lehrer 1998).

In tammar seven cathelicidin genes MaeuCath1-7 have been identified and found to posses the generic cathelicidin features including the signal peptide, a middle cathelin-like region, a valyl130 elastase cleavage site (Zanetti 2004) and a hydrophobic C-terminus (Daly, Digby et al. 2007, Lefevre, Digby et al. 2007). The putative antimicrobial region of all the MeauCaths was rich in arginine and lysine, accounting for 38.9% and 22.9% of the amino acid residues in MaeuCath1 and MaeuCath3 respectively, implying that they are likely to form a strongly amphipathic interaction with membranes.

Although cathelicidins are mostly associated with neutrophils where they are stored in granules (Ramanathan, Davis et al. 2002), constitutive expression of cathelicidin genes has
been reported in various mammalian tissues including kidney, liver and lymphoid organs (Bals, Wang et al. 1998, Wu, Zhang et al. 1999) where they perform different functions.

The sequence heterogeneity of the antimicrobial domain between species has been proposed to be an adaptation to the extremely diverse pathogenic challenge for the various host animals. This point of view gains credence from the tendency in mammals to have more than one cathelicidin gene (Castiglioni, Scocchi et al. 1996, Wu, Zhang et al. 1999, Daly, Digby et al. 2007, Wang, Wong et al. 2011), each presumably targeting different microbes. There is increasing evidence however to show that apart from their antimicrobial role, cathelicidins play significant physiological roles in mammals (Yang, Biragyn et al. 2004). Cathelicidins have been shown to play chemotactic roles (Huang, Ross et al. 1997, Agerberth, Charo et al. 2000), neutralise endotoxins (Golec 2007), enhance re-epithelialisation of dermal wounds (Yang, Biragyn et al. 2004) and modulate angiogenesis (Li, Post et al. 2000).

One of the earliest developmental pointers to the role of cathelicidins in neonatal antimicrobial defence is the presence of cathelicidins in vernix caseosa, the lipid-rich substance that covers a new-born baby and which is thought to have antimicrobial properties (Haubrich 2003, Tollin, Bergsson et al. 2005, Visscher, Narendran et al. 2005). In tammar one cathelicidin gene, MaeuCath1 has been shown to be expressed in the spleen, liver, skin, bone marrow, thymus and gut of the pouch young (Daly, Digby et al. 2007, Lefevre, Digby et al. 2007) and the adult mammary gland (Lefevre, Digby et al. 2007, Khalil, Digby et al. 2008). The secretion of cathelicidins in milk (Joss, Molloy et al. 2009) therefore must raise questions as to whether milk performs multiple roles in the young and perhaps the mammary environment of the mother.
1.8 Hormonal control of lactation

Both the development and function of the mammary gland in all mammals is under hormonal control, the only major difference between species being the requirement of the individual hormones in the various phases of mammary development and function (Topper and Freeman 1980). There is consensus that from embryonic mammary development up to and including lactogenesis, different combinations of the hormones insulin (I), progesterone (pg), 17-β-estradiol (E2), androgen, cortisol, thyroid hormones (T3 and T4), growth hormone (GH), choriomammotropin and prolactin (P) must be present (Topper and Freeman 1980).

1.8.1 Progesterone

Progesterone has been associated with the overt differentiation of the mammary epithelium in early pregnancy that is necessary for alveolar growth. Studies in pubertal mice showed that
progesterone may initiate differentiation by sensitizing epithelial cells to insulin (Topper and Freeman 1980) which in addition to its metabolic regulatory role is required for mammary epithelial cell differentiation. Progesterone is well known for its role as a negative modulator of lactogenesis in several mammalian species since its circulating levels remain high during pregnancy but decline sharply at parturition when secretion of milk is initiated (Kuhn 1977, Topper and Freeman 1980, Nicholas, Simpson et al. 1997). However, this role remains a subject of dispute since the administration of progesterone after the induction of lactation neither diminishes lactation nor down-regulates the expression of individual milk proteins (Topper and Freeman 1980, Nicholas and Tyndale-Biscoe 1985). In eutherians, this diminished response of the lactating mammary gland to progesterone treatment has been attributed to decreased expression of the progesterone receptor (PR) during lactation (Topper and Freeman 1980, Shyamala, Schneider et al. 1990) but it is not clear whether PR expression affects marsupial lactation in a similar way. Further, in a rat explant culture progesterone appeared to have prolactational properties, inducing the expression of alpha-lactalbumin in explants from virgin mammary glands (Nicholas, Sankaran et al. 1981). Whether or not progesterone inhibits lactation, at least it is evident that while it is physiologically relevant during pregnancy, it becomes redundant during lactation and its synthesis either stops or it is rapidly converted to 20α-hydroxyprogesterone (Ray, Averill et al. 1955, Wiest, Kidwell et al. 1968, de la Llosa-Hermier, Lebouelleux et al. 1983, Farina, Ribeiro et al. 2004).

1.8.2 Prolactin

Prolactin is the only hormone without which lactation cannot proceed whether *in-vivo* or *in-vitro* in most mammalian species (Topper and Freeman 1980, Nicholas and Tyndale-Biscoe 1985, Cregan, Mitoulas et al. 2002). Although the combination of cortisol, insulin and prolactin has been shown to be required for lactogenesis *in vivo* (Bird, Hendry et al. 1994) experiments using wallaby mammary explants showed that accumulation of the milk protein
\(\alpha\)-lactalbumin can be achieved \textit{in-vitro} in the absence of extraneous insulin and cortisol as long as prolactin was available at physiological concentrations (Nicholas and Tyndale-Biscoe 1985). Intriguingly, in the wallaby the level of prolactin does not change significantly peripartum (K. R. Nicholas 1995) raising questions of whether the endocrine system only triggers lactogenesis but sustained lactation requires both hormones and autocrine factors intrinsic to the mammary gland. Indeed factors apart from the endocrine system, including the sucking stimulus provided by the neonate and endocrine-independent maternal control have been investigated (Bird, Hendry et al. 1994, Nicholas, Simpson et al. 1997, Hendry, Simpson et al. 1998).

1.8.3 Estradiol

Immediately before parturition, the circulating concentration of eastradiol (E_2) increases (Yoshinaga, Hawkins et al. 1969, Hori, Ide et al. 1970, Horikoshi and Wiest 1970) leading to the suggestion that E_2 may play a role in post parturition MEC proliferation in preparation for milk production. In tammar, 8-12hr postpartum there is an increase in circulating E_2 presumably in order to initiate ovulation (Tyndale-Biscoe, Hinds et al. 1983, Harder, Hinds et al. 1985). Similar to progesterone however, the role of E_2 in mammary function may be limited to pre-parturition morphogenesis and MEC differentiation since ovariectomy does not have a profound effect on lactation in a variety of species (ref). Treatment of pubertal mice with a combination of growth hormone (GH) and E_2 has indeed been shown to enhance ductal morphogenesis.

1.8.4 Growth Hormone

Growth hormone (GH) has been reported to promote the development and functional differentiation of mammary tissue during the lactation cycle of mammals of all classes (Forsyth 1986). In lactating women GH promotes galactopoiesis (Milsom, Breier et al.
1992) and in mice it has been shown to stimulate lobulo-alveolar growth in anterior pituitary-ablated mice suggesting that GH may play a supplementary role to pituitary hormones in mammary morphogenesis.

1.8.5 Insulin in lactation

The array of target organs and tissues on which insulin exerts its regulatory effects includes the majority of mammalian tissues, the most studied among them being the skeletal muscle where insulin stimulates glucose up-take and metabolism and the adipose tissue where it plays a role in lipid metabolism (Dent, Lavoinne et al. 1990). In the mammary gland, studies have shown that the expression of milk protein genes by epithelial cells responds to insulin depending on the stage of the lactation cycle. In mice, MEC’s from virgin animals are insensitive to insulin but during pregnancy they get sensitized and remain responsive throughout lactation. Upon milk stasis they revert to their insensitive state (Oka, Perry et al. 1974) thereby highlighting the significance of insulin signalling in milk synthesis. Studies on tammar mammary explant cultures of tissues from late gestation showed that in order for the tissue to express the full complement of milk proteins insulin must be supplied (K. R. Nicholas 1995). Interestingly the responsiveness of mammary epithelial cells to insulin appears to be independent of insulin receptors since no significant difference has been observed in the capacity to bind insulin between cells from virgin and pregnant animals (Topper and Freeman 1980) implying that the effect of insulin may be mediated by post-receptor mechanisms.

1.8.6 Cortisol

Although cortisol is required for optimal ductal branching in the virgin state and inter-ductal alveolar formation during pregnancy, both these activities have been shown to proceed albeit not optimally in the absence of the hormone (Topper and Freeman 1980). In the mouse,
cortisol has been shown to enhance the formation of the rough endoplasmic reticulum (RER). In the wallaby, the lactogenic effect of prolactin seems to be dependent on cortisol and insulin and a combination of the three hormones is required for the expression of the full complement of milk proteins (Renfree 1984, Nicholas, Simpson et al. 1997). Just like insulin, no significant change in circulating cortisol has been shown to accompany the post-parturition increase in milk production.

1.8.7 Placental lactogen

Similar to prolactin and growth hormone, placental lactogen (PL) is a protein hormone, secreted during pregnancy, that stimulates mammary development and mammary functional differentiation and regulates the metabolic activity of the mother during pregnancy and lactation. However, unlike prolactin and growth hormone, PL appears to be very selectively expressed across mammalian species since bioassay and radioreceptor based detection systems failed to identify PL in several species including horses, armadillos, pigs, dogs, rabbits and the shrews (Forsyth 1986).

Although in humans and other eutherian species PL has been shown to functionally mimic prolactin (Josimovich, Atwood et al. 1963, Handwerger and Freemark 1987) and may play a crucial role in galactopoiesis, it is unlikely that PL plays similar roles in marsupial lactation since mammary gland development and milk secretion appear to proceed normally in the absence of pregnancy (Tyndale-Biscoe 1987).
1.9 Asynchronous concurrent lactation

An intriguing feature of tammar lactation and other macropodid marsupials is that the nursing mother is capable of what is known as asynchronous concurrent lactation (Tyndale-Biscoe 1987, Nicholas 1988, K. R. Nicholas C.J.W 1995, Nicholas, Simpson et al. 1997). At any one time during lactation the wallaby can have a dormant blastocyst and at the same time initiate two concurrent lactations from adjacent mammary glands in order to feed a newly born young and an older sibling at heel simultaneously (Hendry, Simpson et al. 1998). The pouch young feeds on P2A milk produced in the smaller mammary gland while the older sibling feeds on P3 milk secreted in the much larger mammary gland (Nicholas 1988). Several mechanisms have been suggested for ACL all of which agree that the mammary glands
function independently of each other (Nicholas, Simpson et al. 1997, Trott, Simpson et al. 2003).

A study in the agile wallaby (*Macropus agilis*) (Lincoln and Renfree 1981) showed differences in sensitivity to oxytocin between the two mammary glands, the P2 mammary gland being more sensitive than the P3 mammary gland. This study therefore suggested that while the P2 mammary gland is able to respond to mild sucking stimulus of the PY, the P3 gland needs more aggressive sucking provided by the young at heel in order to secrete milk thereby explaining why the larger gland does not produce milk continuously despite the perpetual stimulation of the other gland by the permanently attached PY. The study in *M.agilis* therefore concluded that ACL is regulated by the sucking of the young. Later studies however revealed that ACL not only involves differences in milk volumes but also significant differences in the milk composition of the two mammary glands (Lincoln and Renfree 1981, Nicholas 1988). Further a study in which the intensity of the sucking stimulus was kept constant by repeatedly (every 14 days) fostering 60 day old PY for up to 56 days revealed that the sucking pattern of the young had no effect on milk composition and that the suckled mammary glands progressed to later phase phenotype independent of the sucking stimulus (Trott, Simpson et al. 2003).

Whatever the mechanism, gaining insight into the regulation of ACL has a great potential in improving our understanding of factors intrinsic to the mammary gland which regulate mammary epithelial cell function and lactation. Because of the established roles of the extracellular matrix (ECM) in the mammary gland, this study investigates the potential role of the ECM in regulation ACL.

1.10 The extracellular matrix

The extracellular matrix (ECM) is the complex mesh of proteins that constitute the substratum on which cells rest (Bissell and Barcellos-Hoff 1987). Secreted by various cell
types including fibroblasts, adipocytes, endothelial, myoepithelial and epithelial cells (Jalkanen, Rapraeger et al. 1988, Ekblom, Lonai et al. 2003, Kalluri 2003), the ECM has been viewed as an extension of the cell into its environment and a conduit through which structural and biochemical signals are relayed (Bissell and Barcellos-Hoff 1987).

Since it was first demonstrated that mammary epithelial cells cultured on salivary mesenchyme underwent morphological changes to resemble the salivary gland (Kratochwil 1969, Kratochwil 1971) a large body of evidence has accumulated to show that the stromal environment and particularly ECM regulates the function of tissue specific cells of various organs including the mammary gland. For instance it was discovered that when mesenchymal stem cells were cultured on matrices which mimic the stiffness of brain, muscle and bone they differentiate into neuronal, myoblast, and osteoblast lineages respectively implying that lineage determination may be dependent on the substratum on which the cells attach (Engler, Sen et al. 2006). Similarly, in the mouse model milk protein gene expression is induced when MECs are cultured on floating collagen gels whose elasticity is comparable to the stiffness of the mammary gland but not attached collagen gels which are significantly stiffer (Lee, Parry et al. 1984, Alcaraz, Xu et al. 2008) - mouse β-casein is expressed in cells growing on floating collagen gel and fixed Engelbreth-Holm-Swarm (EHS) matrices but the WAP gene does not express (Lin, Dempsey et al. 1995). In contrast Type IV collagen and fibronectin have no effect on beta-casein expression and heparan sulfate proteoglycan, increased transcription of the β-casein gene but did not induce secretion of the protein (Li, Aggeler et al. 1987).

Against the backdrop of these observations and evidence gained from various in-vitro mammary epithelial cultures Bissel et al have proposed a dynamic and reciprocal relationship between epithelial cells and the ECM. Thus, when a cell comes in contact with ECM, biomechanical and biochemical signals are transduced through ECM receptors on the surface
of the cell to the nucleus resulting in dramatic re-organisation of the cytoskeleton and chromatin structures (Xu, Boudreau et al. 2009). This cascade of signalling events leads to changes in gene expression patterns in the epithelial cell resulting in the secretion of new molecules into the ECM thereby altering the biochemical and biophysical properties of the ECM, in effect initiating another signalling cycle (Bissell, Hall et al. 1982, Bissell and Aggeler 1987, Xu, Boudreau et al. 2009).

ECM constituent proteins are differentially expressed according to the reproductive state and co-regulated with the matrix metalloproteases (MMP’s) which degrade the ECM and the tissue inhibitors of metalloproteases (TIMP’s) which inhibit the MMP’s (Schedin, Mitrenga et al. 2004) and have collectively been associated with histogenic functions including, mammary gland morphogenesis, pubertal ductal branching, lobulo-alveolar formation, lactation and involution (Bissell, Hall et al. 1982, Schedin, Mitrenga et al. 2004). More specifically ECM proteins have been associated with basal/apical polarity of epithelial cells (Paszek, Zahir et al. 2005) and differentiation of mesenchymal stem cells (Engler, Sen et al. 2006), apoptosis, cell survival and proliferation (Butcher, Alliston et al. 2009, Provenzano, Inman et al. 2009). In the developing mouse mammary gland, individual ECM proteins are expressed at specific sites. For example glycosaminoglycans (GAGs) are predominantly expressed on the basal surface of the end bud, while chondroitin sulfate is most abundant in the non-proliferating end bud flank (Silberstein and Daniel 1982). In the cap region where active proliferation is required for penetration during ductal branching, hyaluronate is the predominant ECM protein (Williams and Daniel 1983).

Apart from the major ECM proteins including, laminins, fibronectin, collagens, nidogens and tenascins, the ECM is rich in growth factors which are found cross-linked with ECM proteins by transglutaminases (Nunes, Gleizes et al. 1997). Similarly cytokines are found in the ECM where they are linked to ECM proteins via glycosaminoglycans (GAG’s) and either get
released or remain bound depending on ECM stiffness and degradation of ECM by MMP’s (Discher, Mooney et al. 2009). It is therefore foreseeable that ECM proteins, MMP’s and TIMP’s together, locally regulate mammary function through pathways involving growth factors and cytokine signalling among other mechanisms.

In this study the role of the ECM in local regulation of milk protein gene expression and ultimately ACL was examined. The study hypothesized that treating mammary epithelial cells extracted from tissues in an earlier phase of lactation with ECM from later phases of lactation would change the milk protein gene expression profile of the cells to resemble the phase from which the ECM was extracted. Similarly ECM extracted from earlier phase tissues would de-differentiate later phase cells to adopt the phenotype of the earlier phase. The change in phenotype from one phase to another is determined by measuring the level of expression of the genes for the phase specific markers, tELP, tWAP and tLLP and comparing with the gene for major milk protein β-casein. The study further hypothesizes that the ECM plays a secondary role in the innate immunity of the mammary gland by regulating the expression of cathelicidin genes. To test this hypothesis WallMEC’s from P2A and P2B were cultured on ECM from various phases of lactation and cathelicidin expression determined by RT-PCR.

1.11 Unique opportunities presented by wallaby lactation

Since the phase specific changes in milk composition are accompanied by profound developmental and morphological changes in the neonate, tammar lactation presents unique opportunities to understand the role of individual milk biomolecules in specific facets of neonatal development. For instance since the most prominent delivery of oligosaccharides through tammar milk occurs during the period of greatest brain growth (Green, Newgrain et al. 1980, Nicholas 1988, Menzies and Nicholas 2007), it is feasible that they play a role in
neonatal brain development. Indeed there is increasing evidence suggesting that oligosaccharides found in human milk may enhance neonatal brain development and perform antimicrobial roles among other functions (Bode and Jantscher-Krenn 2012, Jantscher-Krenn and Bode 2012, Jantscher-Krenn, Lauwaet et al. 2012). Similarly the switch from palmitate to oleate as the major fatty acid at the transition from phase 2A to 2B may suggest that oleic acid may be required in developmental changes observed in P2B including vision and hair growth (Renfree, Holt et al. 1982).

1.12 Objectives

**Main Objective:** To investigate the role of the extracellular matrix in mammary epithelial cell function and delivery of domain-specific bioactive proteins (cathelicidins) through wallaby milk

**Specific objectives:**

1. To investigate the temporal regulation of mammary cathelicidins
2. To investigate the antimicrobial properties of milk cathelicidins
3. To investigate the role of cathelicidins in mammary epithelial function
4. To investigate the role of ECM in local control of milk composition
5. To investigate the role of ECM in cathelicidin gene expression

1.13 The overarching hypothesis

The temporal changes in wallaby milk protein gene expression are mediated by progressive changes in the composition of the mammary extracellular matrix through enhanced responsiveness of mammary epithelial cells to lactogenic hormones.
Chapter two

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2.1 Tammar wallaby mammary cathelicidins are differentially expressed during lactation and exhibit antimicrobial and cell proliferative activity

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2.1.1 Abstract

Cathelicidins secreted in milk may be central to autocrine feedback in the mammary gland for optimal development in addition to conferring innate immunity to both the mammary gland and the neonate. This study exploits the unique reproductive strategy of the tammar wallaby model to analyse differential splicing of cathelicidin genes and to evaluate the bactericidal activity and effect of the protein on mammary epithelial cell proliferation. Two linear peptides, Con73 and Con218, derived from the heterogeneous carboxyl end of cathelicidin transcripts, MaeuCath1 and MaeuCath7 respectively, were evaluated for antimicrobial activity. Both Con73 and Con218 significantly inhibited the growth of *Staphylococcus aureus*, *Pseudomonas aureginosa*, *Enterococcus faecalis* and *Salmonella enterica*. In addition both MaeuCath1 and MaeuCath7 stimulated proliferation of primary tammar wallaby mammary epithelial cells (WallMEC). Lactation-phase specific alternate spliced transcripts were determined for MaeuCath1 showing utilisation of both antimicrobial and proliferative functions are required by the mammary gland and the suckled young. The study has shown for the first time that temporal regulation of milk cathelicidins may be crucial in antimicrobial protection of the mammary gland and suckled young and mammary cell proliferation.

**Key words:** cathelicidin, tammar wallaby, mammary epithelial cells, cell proliferation, antimicrobial, lactation
2.1.2 Introduction

Evidence is now emerging to show that milk not only provides appropriate nutrition for the young but also plays a significant role in the development of the mammary gland (Nicholas, Simpson et al. 1997). Major milk proteins, whey acidic protein (WAP) and alpha-lactalbumin for instance have been shown to enhance mammary epithelial cell proliferation (Topcic, Auguste et al. 2009) and initiate apoptosis in mammary cells (Sharp, Lefevre et al. 2008) respectively. In this study we use the unique reproductive strategy of the marsupial, tammar wallaby (*Macropus eugenii*) to investigate the role of mammary-derived cathelicidins MaeuCath1 and MaeuCath7 and their derivative peptides Con73 and Con218, respectively in lactation.

Relative to eutherians, marsupials including wallabies have a short gestation period, leading to the birth of an altricial neonate. The tammar lactation cycle consists of one pre-parturition phase (phase 1) and three post-parturition phases (2A, 2B and 3) during which there is significant remodelling of the mother’s mammary gland, changes in milk composition, and development of the neonate from a foetus-like form to adult-like anatomy (Nicholas 1988, Bird, Hendry et al. 1994, Nicholas, Simpson et al. 1997, Brennan, Sharp et al. 2007). During phase 2A which lasts up to 110 days post-partum, the neonate is permanently attached to the teat and feeds on milk which is low in protein and fat but high in carbohydrates (Nicholas, Simpson et al. 1997). Phase 2B consists of days 100-200 post-partum when the pouch young detaches from the teat and only reattaches to feed. Phase 3 commences approximately 200 days post-partum and is characterised by secretion of high volumes of milk that is rich in protein and fat but low in carbohydrates (Nicholas 1988, Nicholas, Simpson et al. 1997). Development of the respiratory, cardio-vascular and adaptive immune systems in the tammar neonate occurs during lactation and therefore the milk must provide bioactives for immediate
nutritional and immunological demands and to act as signals for development (Basden, Cooper et al. 1997).

A number of genes, including cathelicidins, encode antimicrobial peptides that constitute a significant part of innate immunity in vertebrates (Yang, Biragyn et al. 2004). Cathelicidins belong to a family of proteins generally characterised by an N-terminal putative signal peptide, a conserved cathelin-like domain and a C-terminal heterogeneous antimicrobial domain (Zanetti, Gennaro et al. 1995). Their gene structure characteristically consists of four exons (Gudmundsson, Magnusson et al. 1995), the first three of which encode the pre-proregion (the putative signal peptide and Cathelin-like domain) and the last exon encodes the appropriate protease cleavage site and the C-terminal microbicidal domain. Cathelicidins usually exist in their inactive proform state stored in neutrophil granules. The mature peptide is formed by post-translational cleavage of the proform by different proteases depending on the species. Extrinsic factors, including LPS and LTA have been shown to stimulate mammary epithelial cells (Daly, Mailer et al. 2009) and keratinocytes (Frohm, Agerberth et al. 1997) to express cathelicidin genes.

Numerous functions have been attributed to the two domains of the proform cathelicidin (Yang, Biragyn et al. 2004). The cathelin-like domain is thought to act as a buffer against possible intracellular cytotoxicity of the cationic C-terminal domain (Zanetti, Gennaro et al. 1995) in addition to antimicrobial activity (Li, Post et al. 2000). The human cathelicidin peptide LL-37 has been shown to stimulate colonic mucus synthesis, induce mast cell chemotaxis, suppress neutrophil apoptosis, act as chemoattractant to neutrophils and CD4 T-lymphocytes and neutralise LPS (Yang, Biragyn et al. 2004). Several studies involving LL-37 in various human tissues have demonstrated that cathelicidins play a significant role in wound healing and tissue remodelling. LL-37 enhances re-epithelization of wounds in oral, intestinal, lung, and corneal epithelia implying a possible role in epithelial cell proliferation.
Cathelicidins expressed in the mammary gland during lactation may therefore play a role in mammary tissue remodelling and epithelial cell proliferation.

The structure of a number of tammar mammary-derived cathelicidins has been elucidated and found to consist of typical cathelicidin features (Daly, Digby et al. 2008) but their function in lactation is not understood. MaeuCath1 and MaeuCath7 and their C-terminal peptides Con73 and Con218, respectively are for the first time characterised for bactericidal activity. The effect on the proliferation of tammar mammary epithelial cells (WallMEC) is examined and lactation-phase specific differential splicing investigated.

2.1.3 Materials and Methods

Experimentation with animals

Wallabies were kept in an open yard with adequate vegetation and water. Tissues were obtained following animal ethics approval by the University Animal Welfare Committee (AWC)

RNA purification and RT-PCR

Twenty-six mammary glands were excised for RNA isolation, including two each from non-pregnant (NP) and Phase 1, four from Phase 2A and six each from phases 2B, 3 and weaned (INV) animals. RNA was isolated in two lots of 13 using Tripure Isolation reagent (Roche Diagnostics, Castle Hill, NSW 2154, Australia) according to manufacturer’s guidelines and treated with RQ1 RNase-free DNAse (Promega Corporation). Concentrations of RNA were measured using a Nanodrop 2000 Micro-Volume UV-Vis Spectrophotometer (Wilmington, DE 19810 USA). Reverse transcription was performed using Superscript III™ First Strand Synthesis system (Invitrogen Mount Waverly, Victoria, Australia) with 1μg of total RNA as template. PCR was then performed in two lots of 13 using GoTaq™ DNA Polymerase (Promega). MaeuCath1 primers (5’- ATGCAGGTACTCCTATTGGTG-3’; 5’-
CCCTGGCAGTGGGATAGGAAT-3’ were designed using a tammar mammary gland cDNA library and the draft tammar genome sequence (Lefevre, Digby et al. 2007). Amplification comprised 30 cycles of 94°C 30’, 55°C 40’, 72°C 45’ and a final extension of 72°C for 5 minutes. These primers were designed to amplify a 474bp fragment. To confirm the integrity of RNA and the first strand synthesis product, tammar wallaby GAPDH was amplified using the oligonucleotides 5’-GACTCATGACTACAGTCCATGC-3’ and 5’-GGACATGTAGACCATGAGGTCCAC-3’ under the same cycle conditions. PCR products were loaded onto a 1.2% agarose gel in Tris Acetate/EDTA (TAE) buffer for electrophoresis and band intensity determined using the Image Lab software (Biorad).

Cloning and sequencing of splice variants

RT-PCR amplification products were electrophoresed on 1.2% agarose gel and specific bands excised and purified using the QIAquick Gel Extraction kit (Qiagen, Doncaster, VIC 3108 Australia) according to manufacturer’s guidelines. The purified DNA was cloned into pGEMT Easy (Promega) and sequenced (BD Biosciences, North Ryde, NSW, 2113 Australia).

Peptide design and synthesis

In order to investigate the active domains of both MaeuCath1 and MaeuCath7 proteins, peptides were designed that contained putative active domains. The peptide positive control, CAP18 constitutes the 37 amino acid residues at the C-terminal of the rabbit CAP18 protein [GenBank:AAA31187.1] and has bactericidal activity against Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, and methicillin-resistant Staphylococcus aureus (Travis, Anderson et al. 2000). Peptide Con218 was derived from the heterogeneous carboxyl end of MaeuCath7 sequence (Lefevre, Digby et al. 2007, Daly, Digby et al. 2008) modelled on CAP18 except for an extra lysine at the N-terminal to improve the GRAVY score and
cationicity. Peptide Con73 was derived from MaeuCath1 (Daly KA et al 2008) and modelled on Con218 but was designed to limit the peptide to the region with the highest percentage of cationic residues and best GRAVY score (Figure 3A). Peptides were synthesized (Mimotopes, Clayton Victoria 3168 Australia) utilizing solid-phase chemistry. Peptides were dissolved in PBS at 1mg/ml and stored at -20°C.

Cationicity, hydropathicity and alpha helicity

The grand average of hydropathicity (GRAVY) of peptides was determined by ProtParam software using the Kyte-Doolittle algorithm (Kyte and Doolittle 1982) available through the Expasy Proteomics server. Hydropathicity plots were also generated using ProtParam and window size n=7. Alpha-helicity plots were generated by ProtScale using the Deleage & Roux scale (Deleage and Roux 1987) available at the Expasy Proteomics server.

Bacterial strains and bactericidal assays

All bacterial strains, Pseudomonas aeruginosa ATCC 27853, Salmonella enterica ATCC 43971, Staphylococcus aureus ATCC 29213 and Enterococcus faecalis ATCC 10100 were sourced from American Type Culture Collection (ATCC), Manassas, VA 20108 USA and were a kind donation from Dr. K. Walder (MRU, Deakin University, Australia). Bacteria were streaked onto IsoSensitest (ISA) agar (Oxoid) plates and incubated overnight at 37°C to form isolated colonies. Four individual colonies of each strain were inoculated into 3ml IsoSensitist media for overnight growth at 37°C with agitation on an orbital shaker. The overnight culture was re-inoculated into fresh ISA at a ratio of 1:10 and grown at 37°C until O.D$_{600}$ = 0.6. An empirically determined count of 2000 cells in 80μl ISA were added to each well of a 96 well black wall clear bottom plate (Costar; Corning Incorporated, Corning, N.Y.). AlamaRblue reagent (10μl) and peptides (final concentration100μg/ml) were then added to each well. Either PBS or BSA (100μg/ml) in PBS was added to control wells. A
Florescence reading was measured at time zero (t=0) on Flexi Station II 384 (Excitation=544nm and Emission=590nm) before plates were incubated at 37°C with agitation. Florescence readings were then taken hourly and relative bacterial survival determined by normalising to the PBS treated wells.

**Preparation of cathelicidin conditioned media**

In-frame expressed sequence tag (EST) clones of MaeuCath1 and MaeuCath7 in pCMV.SPORT6 plasmid or empty vector (Invitrogen Corp, Mount Waverly, Victoria, Australia) were used to transfect HEK293T cells at passage number 9 or earlier using the Lipofectamine 2000 (Invitrogen). A pCDNA3.1 plasmid expressing the green fluorescent protein (GFP) was used as a transfection control and success of transfection verified using the IX81 fluorescent microscope (Olympus Imaging Europa GmbH, Hamburg). Media were collected 48 hours post-transfection and filter-sterilised using the 0.2μm Acrodisc syringe filter unit (Pall Corporation) and stored at -80°C until required. Synthetic peptide conditioned media were prepared by adding peptide at the appropriate concentration to media from untransfected wells.

**Purification of cathelicidin protein from conditioned media**

MaeuCath1 sense primer, 5’ATGCAGGTACTCCTATTGGTG3’ was used together with FLAG-tagged antisense primers, 5’CTACTTTGTCATCGTATCCTTTGTAATCCCGAGTGAGGATAGG 3’ and 5’CTACTTTGTCATCGTATCCTTTGTAATCGAATCCCTTCCAGCCCA3’ to amplify full length MaeuCath1 and MaeuCath1b respectively from Phase 2A cDNA. Similarly MaeuCath7 sense primer, 5’ATGCAGGTACTCCTATTGGTG3’ was used in combination with FLAG-tagged antisense primers 5’CTACTTTGTCATCGTATCCTTTGTAATCGCTTGGCGGGGACAGAGAA3’ and
5’CTACTTGTCATCGTCATCCTTGTAATCGTCAATGGTTCCAATGCA3’ to amplify MaeuCath7 and its C-terminal-deleted truncated derivative, tMaeuca7 respectively and PCR products cloned in pTARGET mammalian expression vector (Promega). Sequence-verified pTARGET clones were used alongside an empty vector control to transfect HEK293T cells (ref. section 2.6) in a six well plate and FLAG-tagged protein purified using ANTI-FLAG M2 Affinity Gel (Sigma) according to manufacturer’s instructions. To verify size and purity of recombinant proteins, SDS-PAGE was performed and the gel stained using the Pierce Silver Stain kit (Thermoscientific). Purified protein was used at 100μg/ml in bactericidal assays.

Preparation of tammar mammary cells (WallMEC’s)

Mammary tissue was obtained from tammar wallabies which were confirmed to be pregnant and non-lactating from inspection of the uterus and mammary glands, respectively. Tissue was immediately transferred to 1× Hanks’ Balanced Salt Solution (Sigma Aldridge, Sydney, Australia) with 10 μL/mL penicillin/streptomycin (Gibco, USA) and 2.5 μg/mL Fungizone (Gibco, USA) on ice and transported back to the laboratory for enzymatic digestion to harvest mammary epithelial cells. Mammary tissue was dissected free from fat, weighed, sliced finely and digested with collagenase and hyaluronidase (25 g tissue per 100 mL media) at 37°C for 4 hr. Cells were harvested by filtration (Nalgene filter, 53 μm and 200 μm mesh). The suspension was centrifuged at 80 g /5 min and pellets were washed twice with Hanks Balanced Salt Solution (HBSS). Cell suspensions were centrifuged, resuspended in M199 and 90% FCS/10%DMSO (DMSO-Sigma, Sydney, Australia), and frozen at a density of ~2 × 10⁷ cells/ml.

Culture of WallMEC’s and proliferation assays
Mammary epithelial cells were cultured in growth media (M199/Hams/Hepes media with 1 μg/ml cortisol, 10 ng/ml EGF, 1 μg/ml insulin supplemented with 20% horse serum and 5% foetal bovine serum 1mM glutamine and penicillin/streptomycin). Cells in 100μl of growth media were seeded at 2000/well in a 96 well tissue culture plate (Costar; Corning Incorporated, Corning, N.Y.). After 24hr, 100μl conditioned media was added to each well and cells were maintained at 37°C and 5% CO2 until confluency. Rates of cell proliferation were measured by assaying incorporation of Sulforhodamine B (Skehan, Storeng et al. 1990, Gercel-Taylor, Ackermann et al. 2001, Voigt 2005). Briefly, cells were fixed at days 0, 7 and 11 by addition of 50μl of pre-chilled 50% TCA (wt/vol in water) to each well, incubated for 1hr at 4°C and washed six times with 100μl MilliQ filtered water. The plates were air dried, treated with 0.4% wt/vol Sulforhodamine B (SRB) in 1% acetic acid for ten minutes, washed five times in 1% acetic acid then allowed to air dry. Incorporated SRB was then solubilised in 10mM unbuffered Tris and optical absorbance measured at 515nm on XMark Microplate spectrophotometer (Biorad, Gladsville, NSW, Australia).

Statistical analysis

Statistical analysis of all comparative data was done using the two-tailed t-test with a statistical significance cut-off of 5%.

2.1.4 Results

Alternate splicing of MaeuCath1

Differential splicing of the MaeuCath1 gene was observed during specific phases of the lactation cycle. Two different splice forms, MaeuCath1a and MaeuCath1b were confirmed by sequence analysis and their structure determined by comparison to the genomic MaeuCath1 gene sequence (Figure 1 A and 2). MaeuCath1a is 489bp and comprises all four exons with no intron. MaeuCath1b is 697bp and comprises exons 1 and 2, 256bp of retained intron.
sequence and the 3’untranslated region. The translation stop codon in exon 2 in MaeuCath1b is preceded by 30bp which translate into the peptide sequence VRVSWAGKGF. MaeuCath1a and MaeuCath1b share 100% sequence homology with Maeucath1 and MaeuCath2 which were previously thought to be separate genes (Daly, Digby et al. 2008). Analysis of MaeuCath1 RT-PCR amplicons revealed the two splice variants were differentially expressed in the tammar mammary gland throughout the lactation cycle (Figure 1B). The level of MaeuCath1a transcripts was low in the mammary gland from non-pregnant tammars and at day 16 of pregnancy. However during early lactation MaeuCath1a expression increased to five-fold compared to the non pregnant tammar gland but then declined during mid to late lactation and early involution. In late involution (Day 10), MaeuCath1a expression increased to the same level as the phase 2A gland. MaeuCath1b was expressed at a low level in the non-pregnant (NP) mammary gland, throughout pregnancy and early lactation (day 2 and day 80 lactation). However there was a three-fold increase in MaeuCath1b expression in mid lactation commencing at day 135 lactation. A high level of expression of MaeuCath1b was sustained in the mammary gland throughout late lactation and early involution. At day 10 involution MaeuCath1b expression declined to a level comparable to the non-pregnant gland.

*The grand average of hydropathicity (GRAVY) of cathelicidin peptides.*

The grand average of hydropathicity (GRAVY) of entire cathelicidin peptides is shown in Figure 3. All three cathelicidin peptides, CAP18, Con73 and Con218 had a negative GRAVY indicating they were all hydrophilic. Con73 was the most hydrophilic with GRAVY score of -1.573 followed by CAP18 (-1.097) and Con218 (GRAVY = -0.945). These hydropathicity scores were in agreement with the respective percentages of cationic amino acid residues of 57.7, 37.8 and 31.6.
Unlike GRAVY scores which represent the average hydropathicity of the entire peptide, Kyte and Doolittle plots present hydropathicity scores of individual amino acids computed based on neighbouring residues in a specified shifting window size. Using window size $n=7$ the N-terminal sequence of the longer cathelicidin peptides, CAP18 and Con218 was hydrophilic while the C-terminal was hydrophobic (figure 4). A linear decrease in hydrophilicity from the N-terminal to the C-terminal was observed for CAP18 except for the threonyl$^{35}$, aspartyl$^{36}$ and tyrosyl$^{37}$ residues. The entire Con73 peptide was hydrophilic with the region Gly$^{10}$-Arg$^{16}$ attaining an average Kyte and Doolittle score of -2.01. This hydrophilicity is attributable to the arginine and lysine repeats in this region. Con218 was relatively the least hydrophilic (mean score =-0.83 compared to -1.57 and -1.03 for Con73 and CAP18 respectively).

Deleage-Roux Alpha-helicity plots for the peptides

The alpha-helicity of tamar cathelicidin peptides determined using the Deleage-Roux algorithm and with a cut-off score of 0.99 showed there is significant alpha-helical structure in the N-terminal portion of the longer peptides CAP18 and Con218 (figure 5). There is decreased alpha-helicity in the section spanning residues 15-25 of Con218 and the C-terminal portion of both Con218 and CAP18. The entire sequence of Con73 is above the cut-off score although the middle hydrophilic region had the highest score.

Bactericidal activity of cathelicidin peptides

Synthetic tammar mammary cathelicidin peptides Con218, and Con73 were examined for inhibition of growth of bacterial species (Gram positive bacteria, *Staphylococcus aureus* and *Enterococcus faecalis*; Gram negative bacteria, *Pseudomonas aureginosa* and *Salmonella enterica*) and were compared to the known antimicrobial rabbit cathelicidin peptide CAP18 (Travis, Anderson et al. 2000), over a 4 -16 hour period (figure 6). A BSA control was used
for these analyses and showed no significant inhibition of growth of bacteria. CAP18 acted as a positive control and significantly inhibited the growth of all the four bacterial species tested as expected.

**E. faecalis growth inhibition**

The relative bacterial survival indicated that CAP18 inhibited the growth of *E. faecalis* by more than eight-fold compared to the untreated sample. This level of inhibition was achieved within the first two hours of treatment and extended to sixteen hours of treatment. Peptide Con218 and Con73 had a similar inhibitory effect on *E. faecalis* to CAP18, with a relative bacterial survival value of 0.18 and most of the inhibition within the first two hours of treatment. CAP18 and Con218 however showed marginally but statistically significantly more activity than Con73 (p<0.001).

**S. aureus growth inhibition**

CAP18 inhibited the growth of *S. aureus* by more than eight-fold compared to the untreated sample. Peptide Con73 demonstrated a more rapid effect than CAP18 (relative bacterial survival=0.42 and 0.60 at 2 hours respectively) but a greater activity was observed with CAP18 treatment at the four hour time-point (relative bacterial survival =0.16 and 0.33 respectively). Compared to Con 73 and CAP18, Con218 was significantly less active against *S. aureus* (relative bacterial survival = 0.68, p=0.003).

**S. enterica growth inhibition**

Over a 4 hour period CAP18 and Con218 had an identical inhibitory effect on growth of *S. enterica*. Con73 was relatively slower acting than CAP18 and Con218 but was equally effective after four hours.

**P. aureginosa growth inhibition**
P. aureginosa was the most susceptible bacterial species to cathelicidin peptides. Con73 completely inhibited the growth of P. aureginosa for the 16hr treatment period. CAP18 and Con218 were equally potent with relative bacterial survival of 0.07 and 0.08 respectively.

**Bacterial growth inhibition by full length and truncated MaeuCaths**

In all the bacterial species tested, growth inhibition by full length cathelicidin proteins (MaeuCath1 and MaeuCath7) generally mimicked their derivative C-terminal synthetic peptides. MaeuCath1b and the truncated MaeuCath7 (tMaeuCath7) however did not show significant inhibition of bacterial growth (figure 7b).

**Enhancement of WallMEC Cell proliferation MaeuCaths**

A significant stimulation of proliferation of WallMEC’s was observed when the cells were cultured in growth media supplemented with MaeuCath1, MaeuCath1b and MaeuCath7 (figure 8). The difference in proliferation between MaeuCath1b and MaeuCath1 treated cells did not reach statistical significance (p=0.104). On the contrary tMaeuCath7 did not stimulate growth (figure 8b). By day 7 of treatment cell proliferation of MaeuCath1 and MaeuCath7 treated wells was 7.0% greater than the control. By day 11 treatment, this difference had increased to 9.4% (p=0.006) for MaeuCath1 and 13.3% (p=0.002) for MaeuCath7 treated cells. Peptide Con218 significantly inhibited cell proliferation (15.2% and p=0.026) but by day 11 the proliferation rate was comparable to the control.

**2.1.5 Discussion**

Lactation in the tammar starts with birth of an altricial young and the immune-compromised neonate relies on antibodies and other immune factors secreted in the mother’s milk for defence against microbes (Daly, Digby et al. 2007). The pouch of the tammar wallaby and other marsupials is rich in microflora, including potentially pathogenic bacteria (Yadav, Stanley et al. 1972, Ambatipudi, Joss et al. 2008). Thirty species of bacteria, including
*Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Enterococcus spp* have been isolated from tammar pouches (Old and Deane 1998, Ambatipudi, Joss et al. 2008). The presence of two of these bacterial species, *K. pneumoniae* and *P. aeruginosa* has been associated with mortality of the pouch young in koala (Osawa, Blanshard et al. 1992). Importantly, both *K. pneumoniae* and *P. aeruginosa* and a broad spectrum of other bacterial species are susceptible to the bactericidal activity of cathelicidins from various species (Bals, Wang et al. 1998, Skerlavaj, Benincasa et al. 1999).

The design of antimicrobial peptides from cathelicidins presents a challenge relating to the inter-species heterogeneity of the antimicrobial region. Various attributes of peptide secondary structure including helicity and amphipathicity are however good indicators of peptide efficacy. The extent of amphipathicity and alpha-helicity has been shown to positively correlate with antibacterial activity (Blondelle and Houghten 1992, Johansson, Gudmundsson et al. 1998). High amphipathicity, hydrophobicity and enhanced helical structure are also linked to increased peptide-induced hemolysis (Blondelle and Houghten 1992, Oren and Shai 1996, Oren and Shai 1997, Wieprecht, Dathe et al. 1997, Kondejewski, Jelokhani-Niaraki et al. 1999). This study utilised user-friendly algorithms made available by the ExPaSy server to incorporate the amphipathic and alpha-helical sequence in the peptides.

Computational hydropathicity, amphipathicity and alpha-helicity data obtained for peptides Con73, Con218 and rabbit CAP18 were in agreement with the respective percentages of cationic amino acid residues. The grand average of hydropathicity (GRAVY) scores obtained for all three peptides was negative implying hydrophilicity which complements the hydrophobicity of the C-terminal residues to form an amphipathic structure suitable for interaction with bacterial membranes (Hancock and Sahl 2006). The linear decrease in hydrophilicity from the N to the C-terminal observed in Con218 and CAP18 as deduced from Kyte and Doolittle hydropathicity scores indicated a higher amphipathicity than the less
hydrophobic Con73 (Kyte and Doolittle 1982, Gasteiger, Gattiker et al. 2003). The Deleage-Roux Alpha-helicity plots revealed a lower helicity for Con218 than Con73 and CAP18. The difference in amphipathicity and alpha-helicity between peptides was however not reflected in bactericidal activity probably because all the peptides attained the threshold and demonstrated broad spectrum antimicrobial activity.

This work is the first study to report the antimicrobial activity of mammary-derived marsupial cathelicidins against *S.aureus, E.faecalis, P.aureginosa* and *S.enterica*. Growth inhibition of *S.enterica*, an entero-invasive pathogen and *P.aureginosa* by cathelicidin would be crucial for protection of the young against gut and other systemic diseases. Similarly inhibition of *S.aureus* an agent of mastitis (Siqueira, Aessio et al., Oliver and Mitchell 1983) would provide protection of the mother’s mammary gland. It is paradoxical however that cathelicidin peptides also inhibited growth of *E.faecalis*, a commensal of the gut that is thought to inhibit pathogens including *S.aureus* (Alomar, Lebert et al. 2008). We propose that temporal regulation of cathelicidin synchronized with mother-to-neonate immune transfers as shown in this work, may circumvent this problem.

In the first 48 hours postpartum (Deane et al, 1990) and later on in phase 2B, two immune transfers, marked by an elevated and differential expression of immunoglobulins in milk occur (Daly, Digby et al. 2007). The two periods represent the pouch young’s greatest vulnerability to pathogens hence the need for enhanced protection. In this study we demonstrate that enhanced differential expression of at least one tammar cathelicidin (MaeuCath1a) coincides with the first immune transfer (Daly, Digby et al. 2007) and remains highly expressed until the period preceding the second immune transfer (Daly, Digby et al. 2007). Leading to the second immune transfer there was an estimated five-fold increase in MaeuCath1a transcript. At this stage of neonatal development, cathelicidins would be required to act in synergy with humoral and cellular immune systems to provide protection
from pathogens (Daly, Digby et al. 2007). Following this co-operative microbicidal activity, MaeuCath1a and other tammar cathelicidins would be required to neutralise endotoxins including LPS and LTA (Giacometti, Cirioni et al. 2004) to prevent endotoxic shock. We propose that enhanced MaeuCath1a expression together with immune transfer indicates a possible role of MaeuCath1a in chemotaxis for T cells, neutrophils, mast cells and monocytes which is a typical function of cathelicidins (Yang, Biragyn et al. 2004).

Intriguingly, we observed initiation of MaeuCath1a expression at day 10 involution suggesting that MaeuCath1a may have a role in mammary gland re-modelling during involution. Following milk stasis mammary epithelial cells undergo apoptosis and are cleared through phagocytosis by neighbouring epithelial cells and by macrophages that migrate into the mammary glands (Richards and Benson 1971). The process of milk stasis and involution predisposes the mammary gland to pathogen-mediated mastitis (Oliver and Mitchell 1983). MaeuCath1a may play a chemotactic role to macrophages leading them to the apoptotic epithelial cells and an antimicrobial role protecting the gland from pathogens, hence its up-regulation at involution.

The second MaeuCath1 splice variant (MaeuCath1b) is expressed in all the phases of lactation although a significant up-regulation in expression is observed from mid-lactation to early involution. The intron retention splicing strategy observed in MaeuCath1b eliminates the expression of the 3’ cationic domain leaving only the cathelin-like region. Intron retention is a regulatory mechanism in which the retained intronic sequence is either part of open reading frames (Ner-Gaon, Halachmi et al. 2004) or present in the UTR region (Galante, Sakabe et al. 2004, Ner-Gaon, Halachmi et al. 2004) or sometimes present as the last intron in the transcript. Up to 14.8% of alternately spliced human genes have been reported to have intron retention (Galante, Sakabe et al. 2004) while the frequency in Arabidopsis is as high as 30% (Ner-Gaon, Halachmi et al. 2004).
The abundant expression of MaeuCath1b transcripts is sustained after the timing of immune transfers and the neonate has developed adaptive immunity suggesting it may play a physiological role for the maintenance and proliferation of mammary epithelia during high milk production (Dove and Cork 1989, Bird, Hendry et al. 1994). This is consistent with several studies showing the involvement of cathelicidins in epithelial cell proliferation during wound healing, maintenance and re-establishment of the intestinal barrier integrity and proliferation of lung epithelial cells (Heilborn, Nilsson et al. 2003, Shaykhiev, Beisswenger et al. 2005, Otte, Zdebik et al. 2009). Enhanced WallMEC proliferation following MaeuCath1b treatment in this study supports this hypothesis.

This work further, has ascertained that MaeuCath1a and MaeuCath1b which were earlier reported to be separate genes namely, Ma euCath1 (GenBank: EF624481.1) and MaeuCath2 (GenBank: EF624482.1) (Daly, Digby et al. 2008) are in fact splice variants of the same gene. The study shows that the reproductive strategy of the tammar wallaby can be exploited to show that the cathelicidin gene is temporally regulated by alternate splicing to provide protection for both the pouch young and mammary gland at the time of increased risk of infection and the proliferation of the mammary gland during the time of increased growth of this tissue.
2.1.6 References


2.1.7 Figures

**Figure 1:** Differential splicing of MaeuCath1.

RT-PCR on tammar mammary gland cDNA from various phases of lactation revealed lactation-phase dependent splice variation. **A:** Schematic representation of MaeuCath1 exon structure. All mature mRNA sequence is boxed. Black boxes represent translated sequence. White boxes represent 5’ and 3’ untranslated sequence. **B:** Agarose gel of the amplicons showing relative expression levels of MaeuCath1a and MaeuCath1b amplicons. NP, D16P, 2A, 2B, 3, and INV represent Not Pregnant (well 1), Day 16 Pregnant (well 2), phase 2A lactation, phase 2B, phase 3 and Involution respectively. Phase 2A includes day 2 and day 80 lactation, Phase 2B includes day 135, 150 and 168 lactation. Phase 3 includes day 220, day 235 and day 260 lactation. Involution represents samples collected 1, 5 and 10 days after weaning. Samples in each phase were loaded onto the gel in order of increasing days of lactation.
Figure 2:

Translated sequence of MaeuCath1 splice variants. Panel A represents the translated sequence from the 489bp (MaeuCath1b) variant. Amino acids translated from Exon 1 are marked in black bold letters, Exon 2 in grey box, Exon 3 in black letters and Exon 4 underlined. Panel B represents the translated sequence from the 697bp splice form (MaeuCath1a) which spans exons 1 and 2. The additional 5’ translated sequence of the intron in MaeuCath1a is in plain box.
### A

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Amino acid sequence</th>
<th>ID</th>
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</thead>
<tbody>
<tr>
<td>MaeuCath1</td>
<td>GLTMQVLLLVGLLLSLMTPLGYAQDQPYQDVVLNRFIQEYNTKSESESFLRLSVNLPSQESNDPTAPQLLKFTIRETVCSKSEHRNPCECFKKNGLVEECIGTVDLSSSPSVISCDGPIKVKRGFGKKLRKRRKFRNSIKKRLKNTLNFIKFPVPRQG</td>
<td>[Genbank:ABV019 38.1]</td>
</tr>
<tr>
<td>MaeuCath7</td>
<td>ARALSTKRMRDSTMQVLLVVGLLRLMTSLACAQDQPYQDVVLNRFIQEYNTKSESESFLRLSVNLPPESNDPAPVLLKFTIRETVCPKTEHRNADECFFKNGLVQICGTIDLDSNPSSVISCDFPAKKVKRGLWESLKRKVTKLGDIDIRNTLRNFIKFPVPRQG</td>
<td>[Genbank:ABV019 43.1]</td>
</tr>
</tbody>
</table>

### B

<table>
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<tr>
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<th>Peptide sequence</th>
<th>GRAVY</th>
<th>Percentage Cationic Residues</th>
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<td>CAP18</td>
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</tr>
<tr>
<td>Con73</td>
<td>KVKRFGKKLRKRLKKFRNSIKKRLK</td>
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<td>57.7</td>
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<tr>
<td>Con218</td>
<td>KVKRGLWESLKRKVTKLGDIDIRNTLRNFIKFPVPRQG</td>
<td>-0.945</td>
<td>31.6</td>
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</table>

**Figure 3:**
Amino acid sequence of MaeuCaths and GRAVY scores of cathelicidin peptides. **A:** Partial amino acid sequence of MaeuCath1 and MaeuCath7. Both Con73 and Con218 peptide sequences are shown in grey boxes in their respective parent protein sequence. **B:** The grand average of hydropathicity (GRAVY) of cathelicidin peptides. GRAVY is the computed mean of hydrophobicity and hydrophilicity values for individual amino acid residues. A negative GRAVY indicates hydrophilicity while a positive value indicates hydrophobicity.
Figure 4:

Kyte and Doolittle hydropathicity plots of the peptides. Amino acid position is presented on the $X$-axis. The $X$-axis is scaled to include only those amino acids which fall in a window whose hydropathicity score is computed. Kyte and Doolittle hydropathicity scores (window size $n=7$) for individual amino acids are on the $Y$-axis.
Figure 5

Deleage-Roux Alpha-helicity plots for the peptides. On the $X$-axis is the amino acid residue position. The Deleage –Roux alpha-helicity score is on the $Y$-axis. A cut-off score of 0.99 represents significant alpha-helical structure.
**Figure 6:**
Relative bacterial survival following tammar cathelicidin peptide treatments. Relative bacterial survival (on the Y-axis) was calculated as a fraction of the untreated control. Experiments were performed in triplicate and repeated 3 times. Standard error bars are shown.
Figure 7: Relative bacterial survival following treatment with purified FLAG-tagged MeuCath proteins. A: Silver stained SDS-PAGE gel of FLAG-tagged MaeCaths. i) Vector, ii) MaeuCath1, iii) MaeuCath1b, iv) MaeuCath7, v) tMaeucath7 B: Relative bacterial survival (on the Y-axis) was calculated as a fraction of the untreated control. Experiments were performed in triplicate and repeated 3 times. Standard error bars are shown.
Figure 8.

Wall-MEC cell proliferation. On the X-axis is the duration of treatment in days. On the Y-axis is the absorbance at 515nm of total lysate of SRB-treated Wall-MEC cells. Treatments were done in quadruplicates and experiment repeated twice. Standard error bars are shown.

A: Treatment with synthetic peptides and their MaeuCath parent proteins. B: Treatment with purified FLAG-tagged protein.
Chapter Three

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Ref:

3.1 The extracellular matrix locally regulates asynchronous concurrent lactation in tammar wallaby (*Macropus eugenii*)

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3.1.1 Abstract

Asynchronous concurrent lactation (ACL) is an extreme lactation strategy in macropod marsupials including the tammar wallaby, that may hold the key to understanding local control of mammary epithelial cell function. Marsupials have a short gestation and a long lactation consisting of three phases; P2A, P2B and P3, representing early, mid and late lactation respectively and characterised by profound changes in milk composition. A lactating tammar is able to concurrently produce phase 2A and 3 milk from adjacent glands in order to feed a young newborn and an older sibling at heel. Physiological effectors of ACL remain unknown and in this study the extracellular matrix (ECM) is investigated for its role in switching mammary phenotypes between phases of tammar wallaby lactation. Using the level of expression of the genes for the phase specific markers tELP, tWAP, and tLLP-B representing phases 2A, 2B and 3 respectively we show for the first time that tammar wallaby mammary epithelial cells (WallMECs) extracted from P2B acquire P3 phenotype when cultured on P3 ECM. Similarly P2A cells acquire P2B phenotype when cultured on P2B ECM. We further demonstrate that changes in phase phenotype correlate with phase- specific changes in ECM composition. This study shows that progressive changes in ECM composition in individual mammary glands provide a local regulatory mechanism for milk protein gene expression thereby enabling the mammary glands to lactate independently.
3.1.2 Introduction

In contrast to eutherians, the reproductive cycle of the tammar wallaby (*Macropus eugenii*) consists of a short gestation (phase 1; P1) leading to the birth of an altricial young and a long lactation (Nicholas, Simpson et al. 1997). Tammar lactation has previously been divided into three post-parturition phases; 2A, 2B and 3 (representing early, mid and late lactation respectively) characterised by progressive remodelling of the mammary gland and profound changes in milk composition (Nicholas 1988, Nicholas, Simpson et al. 1997, Daly, Digby et al. 2007, Lefevre, Digby et al. 2007). After milk stasis the mammary gland progresses to involution (INV) and reverts to resemble a pre-pregnancy state. During early and mid lactation the mammary gland is relatively small in size and produces small amounts of milk that is rich in carbohydrates but low in protein and lipids. In contrast late lactation is marked by an enlarged mammary gland secreting large volumes of milk that is rich in protein and lipid. (Nicholas, Simpson et al. 1997).

In the early 1960’s an extreme lactation phenomenon known as asynchronous concurrent lactation (ACL) was described in macropod marsupials (Nicholas 1988). It was observed that at any stage of the reproductive cycle the marsupial could have a dormant blastocyst in the uterus and at the same time effect two parallel lactation cycles producing early and late lactation milk in adjacent glands to support a pouch young and an older sibling at heel concurrently (figure 1A)(Hendry, Simpson et al. 1998). Later experiments showed that apart from the different volumes of milk secreted from adjacent teats, there was differential expression of specific milk protein genes in each mammary gland, suggesting a local intra-mammary mechanism for ACL (Bird, Hendry et al. 1994, Nicholas, Simpson et al. 1997, Brennan, Sharp et al. 2007).
Clearly, paracrine rather than endocrine factors appear to modulate ACL since the observed differences between the individual mammary glands are realised while they are under identical hormonal control. The present study draws from earlier observations that stromal-epithelial interactions are key to mammary differentiation including the study by Cunha et al in which they showed that undifferentiated epidermal cells acquire mammary epithelial phenotype when cultured on mammary mesenchyme (Cunha, Young et al. 1995) and that the proliferative response of mammary epithelial cells to hormones is mediated by the stroma rather than receptors on the epithelial cells themselves (Cunha, Young et al. 1997). Further, complex interactions between the mammary ECM and epithelial cells has previously been associated with changes in the stromal and alveolar structure during lactation (Schedin, Mitrenga et al. 2004, Maller, Martinson et al. 2010). Accordingly, we hypothesized that differences in ECM composition between the adjacent glands was the molecular mechanism regulating ACL.

Mammary epithelial cells (MEC) are attached to ECM whose tissue specific composition and stiffness transduces mechano-signals necessary for modulation of histogenic processes including apical/basal cell polarity (Schedin, Mitrenga et al. 2004, Maller, Martinson et al. 2010) cell proliferation (Provenzano, Inman et al. 2009) and lineage determination of mesenchymal stem cells (Engler, Sen et al. 2006). Bissel et al have described a dynamic reciprocity between the cell and its ECM in which biomechanical signals are transduced through ECM receptors on the surface of the cell to the nucleus resulting in altered gene expression, which in turn leads to altered ECM biomechanical properties (Xu, Boudreau et al. 2009). Importantly, studies have shown that ECM composition dynamically changes under the control of the same effectors that regulate lactation including cytokines, growth factors and hormones (Labat-Robert 2003, Mott and Werb 2004). To understand the role of ECM in the local control of lactation, there is therefore a need to ascertain the lactation-phase specific
changes in mammary gland ECM composition and the subsequent effect on MEC function. In
the present study we utilise the unique multi-phase lactation of the marsupial to analyse
progressive changes in mammary gland ECM and the accompanying alteration in milk
protein expression during the course of lactation. The study utilises ECM from an involuting
mammary gland as a positive control for ECM remodelling. It has been shown in other
models that the ECM undergoes metalloprotease (MMP)-mediated degradation during
involution (Schedin, Mitrenga et al. 2004) and MECs cultured on this matrix undergo
apoptosis.

Each of the phases of wallaby lactation is characterised by the expression of a specific milk
protein gene thereby enabling the quantitation of progressive changes in epithelial phenotype.
The genes for the early lactation protein (tELP), whey acidic protein (tWAP) and late
lactation protein (tLLP-B) are specifically expressed during early, mid and late lactation
respectively (Nicholas, Simpson et al. 1997) whereas the major milk proteins α-casein, β-
lactoglobulin, α-lactalbumin are expressed throughout lactation (Messer and Elliott 1987,
Bird, Hendry et al. 1994, Hendry, Simpson et al. 1998). By culturing tammar mammary
epithelial cells (WallMECs) collected from different phases of lactation on ECM extracted
from mammary glands representing each phase of lactation and measuring the level of
expression of these markers, it was possible to investigate ECM-induced changes of lactation
phase phenotype. The study shows that the ECM regulates the morphology of the alveolar-
like acini which are formed by in-vitro WallMEC cultures and are capable of milk protein
secretion. We additionally show how mammary ECM composition changes during each
phase of lactation and that the ECM ultimately governs phase-specific milk composition
during lactation in the wallaby mammary gland allowing expression of different milk protein
genes and ACL of individual glands.
3.1.3 Results

H&E stain of mammary tissue for ECM extraction

Prior to extraction of ECM from the mammary glands at each phase of lactation, histological sections and the expression of the phase-specific marker genes tELP, tWAP and tLLP-B were examined to confirm the phase of lactation and the integrity of the tissues. In the H&E sections pregnant (PG) mammary tissue had few alveoli surrounded by extensive stroma while early lactation and mid lactation mammary glands had a large number of polygonal alveoli with significant retention of stroma (figure 1B). Late lactation glands had enlarged alveoli with more distended lumina surrounded by less but dense stroma, consistent with earlier observations (Nicholas 1988). The involuting gland (INV) had fewer alveoli in a disordered arrangement within the stroma. The phase specific markers, tELP, tWAP and tLLP-B were expressed exclusively in early, mid and late lactation tissues respectively while β-casein was expressed in all the lactating glands (early, mid, late and INV). GAPDH was uniformly expressed in all the tissues (figure 1C).

Characterisation of acini

In order to investigate the effect of mammary ECM on acini morphology, wallaby mammary epithelial cells (WallMECs) were cultured until they formed acini on ECM extracted from mammary glands representing the different phases of wallaby lactation. Acini were characterised using bright field microscopy and allocated to 6 groups; M1, M2, M3, M4, M5 and M6 in the order of decreasing diameter (figure 2A). Acini were considered mature if they had a smooth outline free of attached cells on the periphery and were greater than 200 \( \mu \text{m} \) in diameter. M1 were the mature acini measuring 200-250 \( \mu \text{m} \). M2, M3 and M4 had free cells attached on their periphery and were 150-200, 100-150 and 50-100 \( \mu \text{m} \) in diameter, respectively. M5 and M6 were acini in the rudimentary stage and measured 25-50 \( \mu \text{m} \) and...
less than 25μm in diameter, respectively. Acini were subsequently stained to identify nuclei using Hoechst 33342 and examined under a confocal microscope to investigate the degree of apoptotic clearance of the lumen. WallMECs derived from all phases of lactation formed 3D acini consisting of a thin layer of cells encapsulating a lumen (figure 2B). The degree of apoptotic clearance of the lumen progressively changed through the various stages of acini maturity (figure 2B). M1 acini were devoid of cells at 0.25 depth into the lumen and M2 at 0.5 depth while there was no strong evidence of existence of a lumen in M3, M4, M5 and M6 acini.

**ECM from non-autologous lactation phase alters acini morphology**

We hypothesized that culturing cells from a lactation phase on ECM extracted from the mammary gland of another phase would alter the phenotype to mimic the phase from which the ECM was extracted. WallMECs from mid lactation were cultured on late lactation ECM and acini morphology and numbers compared to that of acini formed when mid lactation cells were grown on their own phase ECM. On late lactation ECM, acini were more numerous and smaller in size than acini on mid lactation ECM (figure 3), but attained a similar degree of apoptotic clearance of the lumen to M1 type acini. In order to further confirm that changes in acini morphology were caused by the ECM, early lactation cells were cultured on mid lactation ECM and acini compared to those formed when cells were cultured on early lactation ECM. There was no significant difference in the morphology and numbers of acini between early and mid lactation ECM (figure 4). However early lactation cells did not form mature acini on late lactation ECM and involuting ECM (supplementary data).

**Latter phase ECM changes the phenotype of earlier phase WallMEC to resemble the latter phase.**


In order to further investigate the effect of ECM on lactation phase phenotype we hypothesized that culturing earlier phase cells on latter phase ECM would fast-track gene expression to mimic the latter phase phenotype. To test this hypothesis early lactation cells were separately cultured on early and mid lactation ECMS and the expression of the phase specific markers (TELPL and tWAP respectively) measured by isolating RNA from the cells and performing quantitative PCR. Similarly we cultured mid lactation cells on mid and late lactation ECMS and compared the expression of tWAP and tLLP-B (late lactation) between the ECM treatments. The expression of the gene for β-casein was measured as a marker for initiation of lactation (supplementary data). When early lactation cells were cultured on mid lactation ECM they expressed significantly higher levels of tWAP compared to when they were cultured on early lactation ECM (figure 5A). Similarly when mid lactation cells were cultured on late lactation ECM they expressed significantly more tLLP-B (p=0.003; figure 5B) but less tWAP (p=0.005) than when they were cultured on their own phase (mid) ECM.

Earlier phase ECM does not reverse the phenotype of latter phase WallMEC to the earlier phase

We hypothesized that culturing WallMEC from a latter phase on ECM from an earlier phase would reverse milk protein gene expression to the earlier phase phenotype. To test this hypothesis we cultured mid lactation cells on early, mid and late lactation ECM and measured the expression of the gene for tWAP. There was no difference in expression of tWAP between cells cultured on early and mid lactation ECM but tWAP expression was significantly lower on late lactation ECM (figure 6A).

The ECM changes WallMEC phenotype forwards in a step-wise manner

In order to confirm whether the ECM changes the phase phenotype of WallMEC in a sequential manner, early lactation cells were cultured on early, mid and late lactation ECM
and the expression of tLLP-B measured. There was no significant difference in tLLP-B gene
eexpression between early lactation cells cultured on mid or late lactation ECM and their own
phase (early lactation) ECM (figure 6B).

**ECM protein composition is different in each lactation phase**

Individual ECM proteins have been shown to differentially elicit changes in milk protein
gene expression in mammary epithelial cells (Li, Aggeler et al. 1987, Lin, Dempsey et al.
1995). In the present study we hypothesized that the differential expression of milk protein
genes observed after treating cells with ECM from different lactation phases was a result of
changes in the protein composition of the ECM during transition from one lactation phase to
another. Accordingly, ECMs from PG, early, mid and late lactation and involuting mammary
glands were resolved by SDS-PAGE, silver-stained and differentially expressed bands
excised for mass spectroscopy. The larger molecular weight (≥ 250kDa) proteins did not
resolve efficiently leading to multiple identities for individual bands (figure 7A). Differential
expression of ECM proteins from the various lactation phases was observed. Although all the
major classes of basal lamina proteins (collagen IV, laminins, heparan sulphate proteoglycans
and nidogens) were represented in all the ECMs, the multimeric high molecular weight
molecules were more abundant in the lactating gland ECMs than INV ECM. The bands
corresponding to the mix of laminin 1 and tenascin and the mix of heparan sulphate
proteoglycan core protein and glycogen IV were at least twice as dense in PG, early, mid and
late lactation as observed in INV ECM while fibronectin could not be detected by silver
staining in the INV ECM.

Conversely, low molecular weight, monomeric or breakdown products of the major ECM
proteins including nidogen-1 precursor, byglycan, laminin 1 and decorin-like isoform 1
migrating at lower than 24 kDa were predominantly present in late lactation and INV ECM.
Fragments of vimentin and alpha-actinin which are mainly cytoskeletal proteins co-purified
with ECM and were predominantly expressed in early and mid lactation ECMs. Bands which were unique to early and mid lactation and involution (marked with a red ellipse) and migrating at 60, 42 and 15kDa respectively were observed but their identity could not be established using either Swissprot or the wallaby database (figure 7A).

Matrix metalloproteases are differentially expressed during lactation

In order to establish whether the progressive variation in the ECM protein profiles correlates with differential expression and activity of matrix metalloproteases (MMP’s) we performed gelatin zymography using cleared tissue homogenates of pregnant, early, mid and late lactation and involuting mammary glands. Gelatinolytic activity (attributed to matrix metalloproteinase-2) was detectable as latent and/or active forms in mid, late and involution phases of lactation (figure 7B). Cleaved MMP activity was observed to be significantly higher in late lactation and involution and absent in the earlier phases (figure 7 C).

3.1.4 Discussion

The regulation of asynchronous concurrent lactation (ACL) in macropod marsupials has remained perplexing for the reason that two adjacent mammary glands under the same systemic control not only differ significantly in size but produce milk with profoundly different composition (Lincoln and Renfree 1981, Nicholas 1988, Nicholas, Simpson et al. 1997). Several mechanisms have been suggested for ACL (Lincoln and Renfree 1981, Nicholas and Tyndale-Biscoe 1985, Nicholas, Simpson et al. 1997) but ultimately there are two crucial requirements for ACL; firstly, for ACL to be possible the mammary glands have to function independently of each other and be intrinsically controlled (Nicholas 1988, Nicholas, Simpson et al. 1997) and secondly, for ACL to involve such profound differences not only in milk composition and volume but also mammary morphology, the underlying
autocrine regulation is likely to be exerted on the whole mammary gland including, epithelial, myoepithelial and stromal cells.

Clearly, the overt differentiation of mammary tissue, expression of individual milk protein genes and ultimately, secretion of milk in all mammals cannot proceed in the absence of maternal hormones (Ono and Oka 1980, Topper and Freeman 1980, Nagamatsu and Oka 1983, Silberstein 2001). Both in-vivo and in-vitro lactation models require at least one of the several different combinations of insulin (I), progesterone, growth hormone (GH), prolactin (P), cortisol (F), thyroid hormones (T3 and T4) and β17-estradiol (E2) in order to function (Turkington and Hill 1969, Bolander and Topper 1981, Nagamatsu and Oka 1983, Nicholas and Tyndale-Biscoe 1985, Terada, Wakimoto et al. 1988). However, although hormones are required for the expression of tELP (Simpson, Shaw et al. 1998), tWAP (Simpson, Ranganathan et al. 2000, Topcic, Auguste et al. 2009) and tLLP (Nicholas, Simpson et al. 1997, Trott, Wilson et al. 2002, Trott, Adams et al. 2005), available evidence suggests that their differential expression in-vivo is unlikely to be directly regulated by hormones (Nicholas 1988, K. R. Nicholas C.J.W 1995, Nicholas, Simpson et al. 1997, Hendry, Simpson et al. 1998), leading to the proposition that ACL is initiated by altered responsiveness to hormones by the individual mammary glands and that the hormones may play a permissive role. It has indeed been demonstrated in the agile wallaby (Macropus agilis) that differences in milk volumes between the mammary glands may result from progressive changes in sensitivity to oxytocin (Lincoln and Renfree 1981). Accordingly, deciphering differences, whether structural or biochemical, between mammary glands in various phases of lactation has the potential to reveal the effectors of ACL.

The role of mammary ECM in lactation is well documented and includes modulation of epithelial cell growth (Wang, Ballestrem et al. 2011) and differentiation (Streuli 2009), regulation of specific milk protein expression in various eutherian species (Bissell, Hall et al.
1982, Wicha, Lowrie et al. 1982, Li, Aggeler et al. 1987), determination of stromal content (Bissell and Aggeler 1987) and modulation of mammary myo-epithelial cell function (Deugnier, Faraldo et al. 1999). Due to temporal changes observed in the distribution of major mammary ECM proteins during the menstrual cycle (Ferguson, Schor et al. 1990, Ferguson, Schor et al. 1992) the ECM has also been suggested to mediate the response of mammary epithelial cells to hormones.

The present study reports for the first time the involvement of ECM in the phase-to-phase transition in tammar lactation and its possible role in ACL. We observed that the tendency to form mature luminal acini, by both early and mid lactation WallMECs was greatest when the cells were cultured on pregnant, early and mid lactation ECMS and greatly diminished when the cells were cultured on INV (involuting gland) ECM, the latter only supporting the formation of rudimentary acini. In late pregnancy and early lactation the mammary gland undergoes extensive alveolar development (Nicholas, Simpson et al. 1997), as observed in the tissue cross sections, necessary for lactation and therefore the ability of pregnant, early and mid lactation ECMS to support formation of mature acini is a pointer to the significant role the ECM must play in the transition from one phase of lactation to the next. Similarly the inability of INV ECM to support formation of mature acini is consistent with apoptotic activity that characterises involution leading to decreased numbers and size of alveoli (Wicha, Lowrie et al. 1982, Debnath, Mills et al. 2002, Schedin, Mitrenga et al. 2004, Brennan, Sharp et al. 2007, Schedin, O'Brien et al. 2007, Sharp, Lefevre et al. 2007, Khokha and Werb 2011) as observed in this study. However, it is not clear from this study whether acinar size is dependent on the ECM or a factor extrinsic to it since mid lactation cells formed functional acini on late lactation ECM but they were much smaller and more numerous than those formed on other ECMS.
Concordant with dynamic reciprocity between cells and their ECM, in this study we observed that ECM extracted from a late lactation mammary gland altered mid lactation cells to adopt a late lactation phenotype that included mature acini expressing β-casein and tLLP-B. Since previous studies have reported an increase in circulating prolactin at the on-set of late lactation, accompanied by the induction of tLLP-B expression (Nicholas and Tyndale-Biscoe 1985, Nicholas 1988) there was a need to show that the observed increase in tLLP-B was caused by the ECM and not prolactin. Accordingly, in the current study, media on test samples were uniformly supplemented with the full complement of lactogenic hormones (IFPT₃E₂) at concentrations known to induce maximal milk protein synthesis (Topper and Freeman 1980) and control media were supplemented with IF only. These hormone concentrations failed to induce tLLP-B gene expression when cells were treated with ECM from other phases of lactation leading us to conclude that tLLP-B was specifically induced by culturing WallMECs on late lactation ECM. This was consistent with an earlier study in which elevation of prolactin from a physiological concentration of 20ng/ml to as high as 200ng/ml failed to induce expression of tLLP-B in a tammar mammary explant culture using tissue from pregnancy (Nicholas 1988, Trott, Wilson et al. 2002, Trott, Simpson et al. 2003).

Equally noteworthy, when mid lactation WallMECs were cultured on pregnant, early and mid lactation ECMs they expressed at least four times (p≤0.0001) more tWAP than when they were cultured on late lactation and INV ECM without a significant difference in the level of β-casein expression between the treatments. This specific regulation of tWAP expression by ECM is consistent with reports which showed that mouse MEC’s only express WAP when cultured on ECM-induced 3-D alveolar structures (Lin, Dempsey et al. 1995) and that the absence of ECM leads to induction of TGFα expression which inhibits WAP expression. Taken together with the ability of mid lactation cells to express tLLP-B when cultured on late lactation ECM, this observation confirmed that the loss of mid lactation phenotype and
switch to late lactation phenotype was mediated by the ECM. Similarly mid lactation ECM changed the phenotype of early lactation cells to resemble mid lactation. We therefore have demonstrated for the first time that ECM effects the phenotypic changes that occur when the wallaby mammary gland transits from one lactation phase to another. Intriguingly, although early lactation ECM caused a small but significant increase in tELP expression in mid lactation cells relative to the IF treated control, implying some degree of dedifferentiation, similar levels of tELP expression were observed when mid lactation cells were cultured on ECMs from other phases. These observations may have resulted from the insensitivity of tELP gene expression to lactogenic hormones as earlier observed by Simpson et al (Simpson, Shaw et al. 1998).

In this study the transition between phases appeared to correlate with the progressive changes in ECM composition of the mammary gland across the lactation cycle. Although the large multimeric proteins (collagen IV, laminins and heparan sulphate proteoglycans) were uniformly represented in all the phases except at involution, their monomeric or breakdown forms were more abundant in late lactation and involution ECM. The appearance of lower molecular weight fibronectin, laminin and nidogen especially at involution is consistent with degradative remodelling of the ECM by ECM metalloproteases (MMP’s) (Schedin, Mitrenga et al. 2004, Green and Lund 2005). We have demonstrated by gelatin zymography that MMP activity is increased during involution which is consistent with other studies showing increased activity of MMP-2, MMP-9 (Watson and Kreuzaler 2011), MMP-14 and MMP-19 (Rabot, Sinowatz et al. 2007). The increased MMP activity during late lactation follows the period of peak milk production and suggests that the mammary gland may be underdoing gradual involution during late lactation. The phenomenon of gradual involution has previously been reported in the tammar wallaby (Khalil, Digby et al. 2011) and eutherians (Peaker and Wilde 1996, Wilde, Knight et al. 1999).
The uniqueness of mammary ECM from each phase is however exemplified by the phase-specific proteins observed in early and mid lactation and involution (here named P2Ax, P2Bx and INVx respectively because they did not return any ID from Swissprot database). Further investigations are required to identify these products and determine their role in ACL.

Notably annexin A2 and serpinH1 which are typically not integral parts of the ECM were co-purified with ECM proteins. Annexin A2 exists at the extracellular surface of endothelial cells and through its interaction with the plasminogen activator system activates MMP’s thus mediating ECM degradation (Hajjar, Guevara et al. 1996). SerpinH1, while primarily an endoplasmic reticulum protein, is involved in post-translational maturation of collagens (Razzaque and Taguchi 1999) by acting as a molecular chaperon. These associations with ECM may explain their presence, especially in INV ECM. However, taken together, this study has shown that a local mechanism mediated by the mammary gland ECM may modulate the transition between the phases of lactation thereby enabling ACL.

3.1.5 Materials and Methods

Animal ethics and handling

Wallabies were fed ad libitum in an open yard with adequate water. Tissues were obtained in strict adherence to animal ethics guidelines provided by the Deakin University Animal Welfare Committee (AWC).

Preparation of tammar mammary epithelial cells (WallMECs)

Mammary tissue was obtained from tammar wallabies at day 80 (early) and 163 (mid) lactation. The tissue was immediately transferred to 1× Hanks’ Balanced Salt Solution (Sigma 55021C) with 10 µl/ml penicillin/streptomycin (Gibco, USA) and 2.5 µg/mL Fungizone (Gibco, USA) dissected free from fat, weighed and sliced finely. Tissue slices were digested with collagenase and hyaluronidase (25 g tissue per 100 mL media) at 37°C for
up to 4 hr and cells harvested by filtration (Nalgene filter, 200µm mesh followed by 53µm mesh). The suspension was centrifuged at 80xg for 5 min and pellets were washed twice with Hanks Balanced Salt Solution (HBSS). Cell suspensions were centrifuged at 80xg for 5 min, resuspended in M199 and 90% FCS (Invitrogen)/10%DMSO (Sigma, D8418), and frozen at a density of $\sim 2 \times 10^7$ cells/ml.

Histology

A mammary tissue sample weighing approximately 0.5g from wallabies at day 17 of pregnancy, day 80 (early), 163 (mid) and 260 (late) of lactation, and day 10 of involution (INV) was fixed in 10% formalin and processed for hematoxylin and eosin (H&E) staining observing standard histology procedures and the sections characterised by bright field microscopy.

ECM preparation

Mammary tissue (100mg) was homogenised and RNA purified using the RNeasy Lipid Tissue Mini Kit (Qiagen) and reverse transcription-PCR (RT-PCR) performed as summarised in section 5.9. After confirming tissue integrity, mammary ECM was extracted as described by O’Brien et al (O’Brien, Fornetti et al. 2010). Briefly, 3g of mammary tissue from the indicated phases of tammar lactation were individually pulverised to a fine powder in liquid nitrogen and homogenised in a high salt buffer containing 2 mM n-ethylmaleimide (NEM), 200 µg/ml phenylmethylsulfonyl fluoride (PMSF) and 2x protease inhibitor (PI) cocktail (Sigma, P8340). The lysates were subsequently enriched for insoluble extra-cellular ECM proteins through a series of centrifugations and high salt and urea extractions. The ECM was aliquoted and frozen at -20°C until required.

WallMECs culture
WallMECs from mid lactation at passage 4 or earlier were cultured on ECM from pregnant (PG), early, mid and late lactation and involuting mammary glands and assayed for changes in tELP, tWAP and tLLP-B gene expression. Briefly, 200μl of ECM was added to each well of a 6 well tissue culture plate (Costar; Corning Incorporated) and incubated at 37°C for 1 hr to allow coating of the wells. Cells were seeded at 5x10⁴ cells per well in growth media (M199/Hams/HEPES media supplemented with 1 μg/ml cortisol (F), 10 ng/ml EGF, 1 μg/ml insulin (I), 1mM glutamine, 20% horse serum, 5% foetal bovine serum and penicillin/streptomycin). The cells were incubated at 37°C/5% CO₂ until they formed mature acini as earlier described (Sharp, Cane et al. 2006), typically 13 days after seeding. Acini were considered mature after attaining a diameter of 200μm or wider and appeared totally dark under bright field. Upon maturity, acini were washed once with 2ml differentiation media (IF) consisting of 2% FBS in growth media without EGF and incubated in IF containing lactogenic hormones, including 0.2μg/ml prolactin, 650pg/ml triodothyronine and 1 pg/ml estradiol (IFPT₃E₂) for 5 days. Control wells were treated with media containing IF.

**Milk protein gene expression**

RNA extraction from WallMECs and cDNA synthesis were performed as earlier described (Wanyonyi, Sharp et al. 2011) and quantitative PCR’s performed using β-casein, tELP, tWAP, tLLP-B and GAPDH primers and the SsoFast EvaGreen (BioRad) method and quantities determined using the CFX manager software (BioRad). Primers were designed from the tammar mammary gland cDNA library and the draft tammar genome sequence (Lefevre, Digby et al. 2007) as follows: β-casein, 5’- CACCAGCTTCTCCTGATTCT-3’, 5’-CGAGAGAGGGCTGGACTTT-3’; tELP, 5’- ATTTACCATCGTTGCCCTCT-3’, 5’-CAGGACAGCATTCCAGAGT-3’; tWAP, 5’- AAACATGAGATGCTGCCAAC-3’, 5’-TGCATTGATCATCTCTGACA-3’; tLLP-B, 5’- CTCTATCCCTCCAGGCTCAGG-3’, 5’-TGAGGTGAAGGGAATCCTC-3’; GAPDH, 5’-
GACTCATGACTACAGTCCATGCCAT-3’, 5’-GGACATGTAGACCATGAGGTCCAC-3’. Amplification comprised 40 cycles of 95°C 45’, 55°C 45’, 72°C 45’ and melt curve performed from 55°C to 95°C in increments of 0.5°C every 45 seconds. β-casein expression was used as a positive control for milk protein gene expression throughout lactation. GAPDH was amplified under identical cycle conditions as a control for RNA quality.

**ECM protein analysis**

The ECM isolated from mammary tissue was thawed in a water bath at room temperature and diluted 10 times to allow estimation of protein concentration using BCA Protein Assay Reagent (Pierce, Product No. 23227) and 100μg protein loaded on either a 7.5% or 12.5% polyacrylamide gel for the resolution of high and low molecular weight proteins respectively. Silver-stained protein bands were excised and subjected to in-gel tryptic digestion (Rosenfeld, Capdevielle et al. 1992) followed by reduction and alkylation (Shevchenko, Tomas et al. 2006). Mass Spectroscopy was performed using Agilent LC/MSD Trap XCT plus and peptide identity was determined from the SwissProt proteomics database using the Mascot MS/MS Ions Search software (www.matrixscience.com). Peptide ID was further confirmed using a tammar wallaby database (Lefevre, Digby et al. 2007).

**Gelatin zymography**

Zymography was performed as earlier described (Raser, Posner et al. 1995). Briefly a hundred milligram of cryo-preserved mammary tissue was homogenised in 2.5ml of homogenisation buffer and centrifuged at 16,000xg/4°C for 20min and protein concentration of the supernatant estimated using the BCA protein assay reagent. The homogenate was diluted to 2μg/μl and 20μl mixed with 5μl non-reducing loading dye (333mM Tris/HCl pH 6.8, 10% SDS, 50% glycerol) and loaded without heating on 8% SDS-PAGE Mini-Protean II gel (Biorad) containing 0.1% v/v gelatin for resolution at 20mA. Following SDS-PAGE the
gel was washed twice in 2.5% triton X-100 in MilliQ water, each wash lasting 1hr and transferred to assay buffer (50mM Tris/HCl pH 7.5, 10mM CaCl₂, 150mM NaCl) for a 16 hr incubation at 37°C with gentle agitation. Following gelatinolytic digestion, the gel was rinsed once in MilliQ water and stained in 0.25% Coomassie Brilliant Blue (R250), 10% acetic acid, 30% methanol for 20min and destained briefly to allow visualisation of digested represented by clear bands corresponding to MMP activity. To estimate the MMP activity, the gel was photographed and unstained bands compared using the Image Lab software (Biorad).

Microscopy

Wallaby MEC’s isolated from early, mid and late lactation mammary tissue were cultured as described in section 5.3. Thirteen days after plating cells, acini were fixed in 4% paraformaldehyde for 16 hr at 4°C, washed twice with PBS, permeabilised in 0.5% triton X100 for 10 minutes and stained with 10mg/ml Hoechst 33342 (Sigma, B 2261) for 90 minutes. Fluorescent images of nuclear stained acini were captured on Leica TCS SP5 confocal microscope (Leica Microsystems) at excitation 352 nm and emission 454nm. The diameter of acini was estimated by extending the microscopic scale across the image in a cross format and a z-stack cross-section performed at different depths of the acini lumen to determine presence of cells in the lumen. Acini were classified based on their diameter into 6 groups, M1, M2, M3, M4, M5 and M6.

Statistical analysis

Statistical analysis of all comparative data was performed using the two-tailed t-test with a statistical significance cut-off of 5%.
3.1.6 References


3.1.7 Figures

**Figure 1:** Phase specific mammary morphology and milk protein gene expression. **A:** *Tammar mammary glands showing asynchronous lactation.* Q represents quiescent and non-lactating mammary gland, P2A and P3 represent mammary glands producing early lactation milk for the pouch young (PY) and late lactation milk for the young at heel respectively. **B:** *H&E stained cross sections of mammary tissue from various phases of lactation.* PG, Early, Mid, Late and INV represent pregnant (day 17 pregnant), early lactation (day 40), Mid lactation (day 168), late lactation (day 260) and Involution (day 10 post weaning) respectively. Alveoli are marked with A while stroma is marked with S. **C:** *Milk protein gene expression of ECM source tissues.* NTC, PG, Early, Mid, Late and INV represent No template control, and cDNA synthesized from pregnant, early, mid and late lactation and involuting mammary glands respectively while tELP, tWAP, tLLP-B and tβ-cas represent...
tammar early lactation protein, whey acidic protein, late lactation protein-B and β-casein, respectively. PCR products were resolved on a 1.2% agarose gel. PCR’s were performed on cDNA from mammary glands obtained on three different days for each lactation phase in order to confirm reproducibility. Representative samples from each phase are shown.
Figure 2: Acini classification according to morphology. A: Acini at 100x magnification under bright field. M1 were classified as mature acini measuring 200-250 μm. M2, M3, M4 have free cells attached on their periphery and are 150-200, 100-150 and 50-100 μm in diameter respectively. M5 and M6 are acini in the rudimentary stage and measure 25-50 μm and less than 25 μm in diameter respectively. B: Hoechst 33342 nuclear stained acini. z-stack
sections were performed at 2.5, 12.5, 25 and 50% depth of acini from the surface. The degree of apoptotic clearance of the lumen can be determined by observing varying degrees of staining of the lumen.
Figure 3: Acini morphology and abundance after culturing mid lactation WallMEC on mid and late lactation ECM. A: 40x magnification of acini after culturing cells for 13 days. Mid and Late represent mid and late lactation ECM respectively. B: Effect of ECM phase on acini morphology. On the y-axis is the number of acini counted for each acini type. Each treatment was performed in triplicate and the experiment repeated three times. Standard error bars are shown. M1-M6 represent the various acini types in order of decreasing size. Due to the significant difference in the numbers of acini subtypes the y-axis scale is specific to each acinus subtype.
Figure 4: Acini morphology and numbers after culturing early lactation WallMECs on early and mid lactation ECM. **A**: 40x magnification images of acini after culturing cells for 13 days. Early and Mid represent early and mid lactation ECM. **B**: Effect of ECM phase on acini morphology. On the y-axis is the number of each type of acini. Each treatment was performed in triplicate and the experiment repeated three times. Standard error bars are shown. M1-M6 represent the various acini types in order of decreasing size.
Figure 5: Milk protein gene expression after culturing early and mid lactation WallMECs on ECM. A: tELP and tWAP expression in WallMEC cultured on early and mid lactation ECM. On the y-axis is the gene expression level expressed as a percentage of
GAPDH expression. Early and Mid represent the lactation phase of the ECM. Samples were either treated with insulin and cortisol (IF) or the hormone cocktail consisting of insulin, cortisol, prolactin, tri-iodothyronine and estradiol (IFPT3E2). The asterisk denotes significant difference (p=0.011) between early and mid lactation ECM. Samples were treated in duplicate and the experiment repeated twice. Standard error bars are shown. **B: tWAP and LLP-B expression in WallMEC cultured on mid and late lactation ECM.** Mid and Late represent the lactation phase of the ECM. The asterisk represents significant difference (p≤0.005) between mid and late lactation ECM.
Figure 6: **A**: *tWAP* expression in WallMEC cultured on early, mid and late lactation ECM. On the y-axis is the gene expression level expressed as a percentage of GAPDH. Early, Mid and Late represent the lactation phase of the ECM. The asterisk denotes significantly lower expression compared to mid lactation ECM. Samples were treated in duplicate and the experiment repeated twice. Standard error bars are shown. **B**: *tLLP-B* expression in early lactation WallMEC cultured on early, mid and late lactation ECM. The y-axis scale is unique for each of the proteins due to the significant difference in their expression levels.
Figure 7: ECM protein composition and differential expression of matrix metalloproteases (MMP’s) during the lactation cycle: A: Silver stained SDS-PAGE of ECMs from mammary glands of various lactation phases. The high molecular weight (HMW) ECM proteins were resolved on a 7.5% gel while the low molecular weight (LMW) were resolved on a 12.5% gel. Resolution of HMW was poor and clear bands were difficult to isolate for mass spectrometry. Only differentially expressed bands from the LMW gel were isolated for mass spectrometry. Proteins that are unique to only certain phases of lactation are shown in ellipses. B: Gelatin zymography of cleared homogenates of mammary tissue. C represents collagenase control, BL is a blank well, PG, Early, Mid, Late and INV represent cleared tissue homogenates from pregnant, early, mid and late lactation and involution respectively. The unstained bands were predicted to be tammar pro-MMP-2 and active MMP-2 based on molecular size. C: Relative activity of MMP-2: MMP-2 activity in the mammary gland of the tammar during all phases of lactation is shown in arbitrary units determined using the Image Lab software.
Chapter Four

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Ref:

4.1 The extracellular matrix regulates MaeuCath1a gene expression

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4.1.1 Abstract

We have previously shown that the gene for MaeuCath1, a cathelicidin secreted in wallaby milk is alternately spliced into two variants, MaeuCath1a and Maeucath1b which are temporally regulated in order to provide antimicrobial protection to the newborn and stimulate mammary growth respectively. The current study investigated the extracellular matrix (ECM) for its regulatory role in MaeuCath1 gene expression. Reverse transcription qPCR using RNA isolated from mammary epithelial cells (WallMEC) cultured on ECM showed that ECM regulates MaeuCath1a gene expression in a lactation phase-dependent manner. Luciferase reporter-based assays and in-silico analysis of deletion fragments of the 2245 bp sequence upstream of the translation start site identified ECM-dependent positive regulatory activity in the -709 to -15 region and repressor activity in the -919 to -710 region. Electrophoretic Gel Mobility Shift Assays (EMSA) using nuclear extract from ECM-treated WallMEC showed differential band shift in the -839 to -710 region.

Keywords:
Extracellular matrix, mammary epithelial cells, cathelicidin, lactation, cathelicidin promoter, gene regulation
4.1.2 Introduction

Cathelicidins and other host defence peptides delivered through milk constitute a significant part of neonatal innate immunity (Armogida, Yannaras et al. 2004) and may regulate the function of mammary epithelial cells. Apart from their primary role as antimicrobial proteins, cathelicidins have been shown to effect the chemotaxis of mast cells, neutralise endotoxins, chemo-attract CD4 T-lymphocytes (Zanetti, Gennaro et al. 1995, Yang, Biragyn et al. 2004) and stimulate mammary cell proliferation in a domain-specific manner (Wanyonyi, Sharp et al. 2011). Therefore understanding how the expression of cathelicidins is regulated during the course of lactation has the potential to provide a platform for strategic delivery of milk bioactives needed for neonatal development and the optimal function of the lactating mammary gland.

However, except for studies which have associated the expression of cathelicidins by immune cells with pathogenic infections (Daly, Mailer et al. 2009), no regulatory factors have been described for cathelicidins secreted in milk. Additionally, the majority of studies on mammalian cathelicidins have been performed in eutherian models (Yang, Chen et al. 2004) in which milk composition does not change significantly after the colostrum phase and the neonate is born precocious, making it difficult to correlate early developmental changes with bioactives in milk.

This study utilises the unique reproductive strategy of the tammar wallaby (Macropus eugenii) to investigate how the expression of the gene for MaeuCath1, a cathelicidin expressed in milk, is regulated. Compared to eutherians, marsupials including tammar wallaby have a short gestation leading to the birth of an immune-incompetent fetus-like young that relies on the mother’s ability to profoundly change its milk composition throughout lactation (Nicholas, Simpson et al. 1997). Tammar lactation is divided into three
post-partum phases (P2A, P2B and P3). During P2A and P2B the milk is rich in carbohydrate but low in protein and lipid, but during P3 the mother produces concentrated milk that is rich in lipid and protein but low in carbohydrates. Interestingly, the wallaby is capable of an extreme lactation phenomenon known as asynchronous concurrent lactation (ACL) whereby the mother simultaneously produces P2A milk from one mammary gland and P3 milk from another in order to feed a newly born pouch young and an older sibling at heel concurrently (Nicholas 1988). Since these differences in milk composition are realised while the two lactating mammary glands are under identical hormonal influence (Nicholas 1988, Nicholas, Simpson et al. 1997), it has been suggested that milk composition is controlled significantly by paracrine factors in addition to endocrine factors. Specifically, the mammary extracellular matrix (ECM) appears to modulate the expression of major milk protein genes in a lactation phase-dependent manner (Wanyonyi In Press).

We have previously shown that the gene for MaeuCath1 is regulated through alternate splicing resulting in two variants MaeuCath1a and Maeucath1b (Wanyonyi, Sharp et al. 2011). The expression of MaeuCath1a, the splice variant containing the antimicrobial region (Wanyonyi, Sharp et al. 2011) is synchronised with periods of increased vulnerability of both the pouch young and the lactating mammary gland to microbial infections. Such periods of vulnerability include the early post-partum period (P2A) when the PY has not developed a competent immune system (Daly, Digby et al. 2007) and late involution when the mammary gland environment resembles a wound-healing process (Oliver and Mitchell 1983, O’Brien, Martinson et al. 2012) and is pre-disposed to potential mastitic infections (Oliver and Mitchell 1983). MaeuCath1b which does not appear to have antimicrobial activity is expressed predominantly during peak lactation and may play a role in the growth of the mammary gland by enhancing mammary epithelial cell proliferation (Wanyonyi, Sharp et al. 2011).
Earlier studies have shown that mammary epithelial cell (MEC) differentiation and gene expression are dependent on temporal changes in the mammary ECM (Bissell, Hall et al. 1982, Schedin, Mitrenga et al. 2004), and therefore we hypothesised that the ECM regulates the lactation phase-specific expression of MaeuCath1 by MEC. The pathogen-induced expression of the human cathelicidin hCAP18 in gingival epithelial cells has indeed been shown to be signalled through the fibronectin-integrin pathway (Ouhara, Komatsuzawa et al. 2006), suggesting that cathelicidins including MaeuCath1 may be regulated by the ECM through enhanced response of epithelial cells to external stimuli.

Therefore the current study investigated the effect of mammary ECM extracted from different phases of lactation on MaeuCath1 gene expression in wallaby mammary epithelial cells (WallMEC).

4.1.3 Results

**Differential expression of MaeuCath1 and effect of ECM**

In order to determine the effect of ECM on MaeuCath1 gene expression WallMEC were cultured on mammary ECM from P2A and P2B in growth media alone (NH) or in the presence of the full complement of lactogenic hormones (IFPT$_3$E$_2$) RNA was extracted for cDNA synthesis and quantitative PCR. A modified image of an agarose gel previously published by us (Wanyonyi, Sharp et al. 2011) showing RT-PCR products of RNA isolated from mammary tissue from P2A and P2B (figure 1A) has been presented alongside RT-PCR expression data for MaeuCath1 in WallMEC in order to compare the *in vivo* and *in vitro* data. MaeuCath1a expression was significantly higher when WallMEC were cultured on P2A ECM than P2B ECM (p=0.002; figure 1B) which is similar to the expression profile observed in mammary tissue. However, both P2A and P2B WallMEC did not express MaeuCath1b irrespective of the substratum on which they were cultured (figures 1B and C). The
expression of MaeuCath1a in both P2A and P2B WallMEC cultured on P2A ECM was significantly higher when they were treated with IFPT$_3$E$_2$ than NH (p≤0.04; figure 1D). A similar effect was observed when P2A WallMEC were cultured on plastic although the expression level was significantly lower than that observed on ECM (p<0.001; figures 1E).

In order to determine that the differential expression of MaeuCath1a on ECM is specific to ECM treatment, P2A WallMEC were grown on a tissue culture plate (plastic) in the absence of ECM and analysed for MaeuCath1 gene expression by qPCR. WallMEC expressed significantly less MaeuCath1a (p≤0.002) when cultured on plastic than on P2A ECM (figure 1B) but 70% higher (p=0.01) than the expression level observed on P2B ECM.

In order to determine whether MaeuCath1a expression in WallMEC is dependent on the lactation phase from which the cells were extracted, P2B WallMEC were cultured on P2A ECM in NH or IFPT$_3$E$_2$ media and RNA isolated for cDNA synthesis and qPCR. MaeuCath1a expression was significantly lower (p=0.001) in P2B than P2A WallMEC when both were cultured on the same ECM (figure 1C).

**Activity of MaeuCath1 promoter fragment -709 to -15.**

In order to determine the putative promoter region for MaeuCath1, the 5'-UTR of the MaeuCath1 gene (Ensembl, Scaffold97912 wallaby genome) was subjected to preliminary *in-silico* studies to locate prominent regulatory elements including the TATA box (figure 2) and the 2245 bp sequence 5’ to the ATG translation start site selected for further investigation. Deletion fragments of the putative promoter region (figure 3A and B) were amplified by PCR using the primers in table 1, cloned in the pGL3 basic (luciferase reporter) vector and sequenced. The deletion fragments were analysed for promoter activity in P2A WallMEC cultured on either plastic, P2A or P2B ECM. Each well was co-transfected with pSV-β-Galactosidase for normalisation of activity data and control wells were transfected with
pMAX-GFP (Amaza) for visual comparison of transfection efficiency by bright field and fluorescent microscopy. The pGL3/Rat β-casein promoter construct (Rβ-cas) was included as a positive control for response of WallMEC to lactogenic hormones. There was no difference in transfection efficiency between cells cultured on the three substrata although by day 2 post-transfection the cells cultured on ECM had started aggregating into pre-acini structures (labelled PA in figure 3C).

When P2A WallMEC were cultured on P2A ECM, fragment -709 to -15 elicited at least 2.5 times higher promoter activity (p≤0.05) than the remaining fragments (figure 4A). Fragment -919 to -15 which includes the -709 to -15 sequence and the 210 bp immediately 5’ to it was 4.3 times less active than the -709 to -15 fragment (p<0.001). Similarly the -484 to -15 fragment which constitutes the 3’ end of the -709 to -15 fragment was 6.3 times less active than the -709 to -15 fragment (p<0.01). When P2A WallMEC were cultured on plastic and P2B ECM, promoter activity for all the fragments was similar to background activity (pGL3 basic) observed when the cells were cultured on P2A ECM (p≥0.4; figure 4B and C). When P2B WallMEC were used, except for the Rβ-cas control which had 13.2 times higher activity (p=0.009) than the pGL3 basic control, all the fragments exhibited background promoter activity irrespective of the substratum (figure 4D-F).

In order to establish whether the promoter activity of deletion fragment -709 to -15 is dependent on lactogenic hormones, P2A cells were transfected with the promoter fragment and treated with either IFPT3E2 or NH and promoter activity measured. Promoter activity was twice as high (p=0.048) in IFPT3E2-treated cells as in NH-treated cells (figure 5A).

**Activity of MaeuCath1 promoter region -919 to -710**

Since the 210 bp sequence immediately 5’ to the -709 to -15 fragment appeared to decrease the activity of the -709 to -15 fragment, we hypothesized that the -919 to -710 (210 bp) had
repressor elements. We also hypothesised that the 225 bp sequence from -709 to -485 (figure 5B) contained positive regulatory elements since it appeared to be the active part of the -709 to -15 fragment. To test this hypothesis the 210 and 225 bp fragments were ligated to pGL3/Rβ-cas immediately 5’ to the casein promoter and the constructs transfected into P2A WallMEC to examine luciferase activity. Fragment -919 to -710 reduced Rβ-cas promoter activity by 1.6 times (p=0.01) while fragment -709 to -485 increased Rβ-cas promoter activity by 1.9 times (p=0.005; figure 5C).

In order to confirm the repressor activity of the region -919 to -710, primers (table 1) were designed so as to clone the promoter regions -2245 to -920 and -1260 to -920 upstream of the active promoter region -709 to -15 in pGL3 Basic vector, in effect reconstructing fragments -2245 to -15 and -1260 to -15 excluding the putative repressor, and constructs (figure 6A) transfected into P2A WallMEC to examine luciferase activity. After exclusion of the putative repressor region, fragment 1260 to -15 had 4.05 times higher (p=0.022) promoter activity than -709 to -15 (figure 6B). Similarly fragment -2245 to -15 had 2.75 times higher (p=0.02) activity than -709 to -15 (figure 6B).

**Putative transcription factor binding sites in the MaeuCath1 promoter**

In order to predict the regulatory elements responsible for the activity of the MaeuCath1 promoter, the entire -2245 sequence was searched against the TRANSFAC proteomics database using P-Match, Patch 1.0 and AliBaba 2.1 software and a plot of the binding sites made to scale (figure 7). Seventy one putative transcription factor binding sites including 23 Specificity protein 1(Sp1), 17 CCAAT-enhancer-binding proteins (C/EBPα), 12 Yin Yang (YY1), 7 Nuclear factor 1 (NF-1), 5 Signal transducer and activator of transcription (STAT5), 4 TG-interacting factor (TGIF), 2 Glucocorticoid receptor (GR) and 1 Activating protein-2 (AP-2) were identified (figure 7). Specific transcription factor binding sites appeared to
cluster in certain regions of the promoter. For example, 8 out of 12 binding sites in the region -2014 to -1515 were C/EBPα-specific while 7 out of fifteen binding sites in the region -514 to -15 were Sp1-specific. Similarly 8 out 12 YY1 sites were found in the region -1165 to -625 while all the TGIF binding sites were found in the region -919 to -710 (figure 6A and 7).

**Putative transcription factor binding sites in the MaeuCath1 promoter region -839 to -710**

In order to identify the regulatory elements present in the putative repressor region -919 to -710, the respective sequence was used to probe the TRANSFAC database as described in section 2.7 and 50 bp overlapping primer pairs designed for EMSA as described in section 4.8 to identify transcription factors by gel shift assay. *In-silico* results showed that fragment -919 to -710 contained one C/EBPα, three YY1, two NF-1 and four TGIF putative binding sites (figure 6A). Although faint band shifts were observed in all nuclear extract/DNA mixes (figure 8), the most prominent band shift occurred when primer pairs GS3F/GS3R, GS4F/GS4R and GS5F/GS5R representing regions -759 to -710, -799 to -750 and -839 to -790 respectively were included in a binding reaction with the nuclear extract from cells cultured on P2A ECM.

**4.1.4 Discussion**

In a previous study (Wanyonyi, In Press), using wallaby mammary extracellular matrix (ECM) extracted from the different phases of lactation, we showed that the switch from the P2A lactation phenotype to P2B and finally P3 is mediated by temporally regulated changes in the ECM composition. Using SDS-PAGE and mass spectrometry we showed that although the large multimeric proteins (collagen IV, laminins and heparan sulphate proteoglycans) were uniformly represented in all the phases except at involution, their monomeric or breakdown forms were more abundant in late lactation and involution ECM. Gelatin
zymography data from the study showed that the changes in the ECM could be mediated by temporal changes in matrix metalloprotease activity during lactation. In a separate study, we reported that the gene for MaeuCath1, a cathelicidin secreted in wallaby milk was alternately spliced into two variants, MaeuCath1a which is expressed in P2A and involution and MaeuCath1b which is expressed during mid to late lactation (Wanyonyi, Sharp et al. 2011). The current study has examined the possible link between the ECM and the temporally regulated expression of MaeuCath1 by first examining the expression of MaeuCath1 in WallMEC cultured on ECM from different phases of lactation and then performing luciferase reporter-based assays on WallMEC cultured on ECM and transfected with MaeuCath1 putative promoter fragments.

MaeuCath1a was highly expressed in cells cultured on P2A ECM but poorly expressed on P2B ECM which is similar to mammary tissue data (Wanyonyi, Sharp et al. 2011). Equally noteworthy, cells cultured on plastic expressed significantly less MaeuCath1a than those cultured on P2A ECM, consistent with a large number of studies in which mammary epithelial cells did not attain cyto-differentiation and secretion of milk proteins when cultured on plastic (Bissell and Ram 1989, Talhouk, Neiswander et al. 1998, Rose, Aso et al. 2002) and highlighting the possible role of the ECM in the temporal regulation of MaeuCath1 expression. However the failure of P2B cells to express MaeuCath1a when cultured on P2A ECM, implies that ECM-induced cyto-differentiation of WallMEC may be directional and once the cells have acquired a later phase phenotype they cannot be programmed backwards to an earlier phase by the ECM. We have indeed observed that while P2B ECM can program P2A cells to express tammar whey acidic protein (tWAP), a marker for P2B, the ECM from P2A does not program P2B cells to express the early lactation protein (tELP), the marker for P2A (Wanyonyi, In Press). This would similarly explain why P2A cells retained their
phenotype (expressing MaeuCath1a) when they were cultured on P1 ECM (supplementary data).

Changes in the biochemical and biomechanical properties of the ECM that surrounds mammary epithelial cells initiate the transduction of signals into the nucleus of the cells causing changes in the chromatin structure, altered gene expression and ultimately another cycle of change in the ECM (Bissell, Hall et al. 1982, Nicholas and Tyndale-Biscoe 1985, Nicholas, Collet et al. 1991). However, it is apparent that for the ECM-cell crosstalk to mediate epithelial cell polarity and milk protein gene expression, there has to be a synergistic involvement of lactogenic hormones (Topper and Freeman 1980, Bissell and Aggeler 1987). It is not surprising therefore that when WallMEC were cultured on P2A and INV (involuting mammary gland) ECM in the presence of IFPT$_3$E$_2$, they expressed more MaeuCath1a than the NH treated cells (supplementary data) and the activity of promoter fragment -709 to -15 was enhanced by IFPT$_3$E$_2$. However the reason for the lack of expression of the second variant (MaeuCath1b) is unclear and requires further investigation. We postulate that the splice site preference in vivo is different from the selection in vitro as has been observed for the mouse kappa-immunoglobulin precursor in which the in vivo splicing mechanism preferentially selected the 5’-most donor splice site whereas such selectivity was lost in vitro (Perry, Kelley et al. 1980, Lowery and Van Ness 1988). Other complex splice site selection mechanisms have been suggested to be responsible for the expression of different variants in vitro and in vivo (Aebi, Hornig et al. 1986).

The ECM modulates milk protein gene expression through transcription factors which recognise regulatory elements on milk protein gene promoters. For instance studies have shown that β-casein expression is signalled by the binding of laminin I to β1-integrin through the integrin-linked kinase (ILK) –mediated activation of Rho GTPase and Rac1 (Akhtar, Marlow et al. 2009). In the presence of prolactin, Rac 1 then activates the PrlR/STAT5
signaling cascade in effect recruiting STAT5 which drives β-casein transcription (Wakao, Gouilleux et al. 1995). Therefore transcription factor binding sites present on the ECM-responsive MaeuCath1 promoter fragment -709 to -15 and the proximal -919 to -710 sequence which has repressor properties are of particular interest with regard to cathelicidin expression and how it is regulated by the ECM. Transcription factor binding sites on the DNA fragment -709 to -15 showed there were 10 Sp1, 4 STAT5, 3 YY1, 3 C/EBPα and 1 NF-1 sites while the putative repressor region -919 to -710 had 10 transcription factor binding sites 4 of which were TGIF, 2 YY1, 2 NF-1, 1 C/EBPα and 1 Sp1.

Through binding to the highly ubiquitous GC boxes Sp1 positively regulates a large number of genes (Li, He et al. 2004) but it is especially prominent in myeloid cell differentiation in which, together with C/EBP it activates individual genes including lactoferrin (Khanna-Gupta, Zibello et al. 2000) and would probably be playing a similar role in MaeuCath1 expression. The link between Sp1 and the ECM has been demonstrated in several in vitro studies including one in which silencing of Sp1 down-regulated collagens, TIMP-1 and decorin (Verrecchia, Rossert et al. 2001) and another in which together with NF-1, Sp1 was shown to enhance COL1A1 promoter activity in Tsk2 fibroblasts (Christner, Hitraya et al. 1998). However a feedback effect of ECM on Sp1 activity needs to be established in order to link ECM regulation of MaeuCath1 to Sp1. Similar to Sp1, YY1 is ubiquitously expressed but it is a context-dependent regulator of transcription (Gordon, Akopyan et al. 2006) that can not only initiate transcription, but also activate (Guo, Casolaro et al. 2001) and repress (Shi, Lee et al. 1997, Sui, Affar el et al. 2004) gene expression. In the context of MaeuCath1 expression it is feasible therefore to suggest that YY1 may determine the phase-specificity of its expression.

Interestingly, the regulatory elements found on the active promoter fragment -709 to -15 have been shown to exist in clusters known as composite regulatory elements (CoRE) acting
together to regulate milk protein genes. For example the CoRE in whey protein gene promoters includes NF-1, glucocorticoid receptor (GR) and STAT5 while casein promoters have STAT5, YY1, GR and C/EBP binding sites (Groner, Altiok et al. 1994, Rosen, Zahnow et al. 1998). Therefore it appears that the overall function of the regulatory elements on the -709 to -15 fragment is to activate the expression of MaeuCath1 through the synergistic effect of the ECM and lactogenic hormones hence the elevated promoter activity. Conversely the clustering of TGIF binding sites on the putative repressor sequence -919 to -710 suggests that this region may be responsible for the temporal down-regulation of MaeuCath1a expression during P2B and P3 since TGIF is a potent repressor of transcription (Wotton, Lo et al. 1999, Liu and Huang 2008, Melhuish, Chung et al. 2010).
4.1.5 References


4.1.6 Materials and Methods

Animal ethics and handling

Tammar wallabies (*M. eugenii*) were reared in an open grass yard where they had free access to adequate shelter and water and were fed *ad libitum*. Animal ethics guidelines provided by the Deakin University Animal Welfare Committee (AWC) were observed.

Isolation of tammar mammary epithelial cells (WallMEC)

Wallabies at day 21 and 136 lactation representing P2A and P2B respectively were euthenised and mammary tissue surgically removed and immediately placed in 1× Hanks’ Balanced Salt Solution (HBSS) (Sigma 55021C) containing 10 μg/ml penicillin/streptomycin (Gibco, USA) and 2.5 μg/mL Fungizone (Gibco). After removal of fat, the tissue was weighed and sliced repeatedly into tiny fragments followed by incubation at 37°C for up to 4 hr in 400 units/ml Collagenase Type 3 and 100 units/ml Hyaluronidase (Worthington) observing a ratio of 25 g tissue per 100 mL digest media and cells harvested by filtration, first through 200μm mesh and then 53μm mesh Nalgene filter. The filtrates were centrifuged at 80xg for 5 min and cell pellets washed twice by suspending in HBSS and centrifuging at 80xg for 5 min. After the final wash, cells were resuspended in freezing media (90% FCS (Invitro Technologies)/10%DMSO (Sigma, D8418), at a density $2 \times 10^7$ cells/ml and frozen in liquid nitrogen.

Preparation of ECM

ECM was isolated from mammary tissue by homogenisation followed by sequential high salt washes and urea extraction as described by O’Brien *et al* (O’Brien, Fornetti et al. 2010). Briefly, previously snap-frozen mammary tissue was added to a ceramic mortar containing liquid nitrogen and pulverised to a fine powder followed by homogenisation in a high salt buffer (1g of tissue in 2ml) containing 2 mM n-ethylmaleimide (NEM), 200 μg/ml
phenylmethylsulfonyl fluoride (PMSF) and 2x protease inhibitor (PI) cocktail (Sigma, P8340). The homogenate was enriched for insoluble ECM proteins by performing a second high salt wash and urea extraction, including a high speed centrifugation step (1 hr at 100,000xg) between the washes and the ECM serially dialysed to remove protease inhibitors and urea. The final dialysis was performed in Hams F12/ M199 media (Gibco®) in the absence of serum and the ECM aliquoted for freezing at -20°C until required.

Investigating the effect of ECM on MaeuCath1 expression

Undiluted ECM (380 - 420 μg/ml) was added to each well of a 6 well tissue culture plate (Costar; Corning Incorporated) so as to completely cover the bottom and the plate incubated at 37°C for 1 hr to allow coating. To the control wells (plastic), growth media containing serum was added without ECM. After coating the wells, WallMEC from P2A and 2B at passage 5 and earlier were seeded at a density of 5x10^4 cells per well in growth media (Hams F12/ M199/HEPES containing 5% foetal bovine serum (FBS), 20% horse serum, 1mM glutamine and 100U/ml penicillin/100 μg/ml streptomycin and cells incubated overnight at 37°C/5% CO₂. Growth media was aspirated from the cells and fresh media containing 2% FBS added to the no hormone (NH) treated samples while the media on the hormone treated (IFPT3E2) wells was supplemented with 1 μg/ml cortisol (F), 10 ng/ml EGF, 1 μg/ml insulin (I), 0.2 μg/ml prolactin, 650 pg/ml triodothyronine and 1 pg/ml estradiol and the cells incubated for a further 2 days. The cells were harvested for RNA purification using the RNeasy protocol (Qiagen) and 1μg of RNA used for cDNA synthesis and RT-PCR using primers shown in table 1.

Cloning of MaeuCath1 promoter fragments

Cloning primers were designed to include Xho I (Forward) or Hind III (Reverse) restriction enzyme sites to allow cloning of MaeuCath1 promoter fragments into the multiple cloning site.
(MCS) of the pGL3 basic vector (Promega) 5’ to the luciferase gene. For cloning promoter fragments without the putative repressor (-919 to -709) the Hind III site in the reverse primers was replaced with a Kpn I site. Promoter fragments were amplified by PCR using wallaby genomic DNA isolated from the mammary gland as template and 30 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 60°C and extension for 1 min at 72°C followed by a final extension at 72°C for 5 min and PCR products purified using the QIAquick kit (Qiagen). Purified PCR products and pGL3 plasmid (10μg each) were double digested with Xho I and Hind III in buffer 2 (New England Biolabs) and gel-purified using the QIAquick Gel Extraction Kit (Qiagen) before ligation of 100 ng of plasmid to 150 ng promoter fragment. Transformation of the ligation mix into JM109 cells and screening for recombinants was done according to standard procedure (Maniatis 1982).

**Luciferase assay**

WallMEC were seeded (without antibiotics) in a 96 well plate so as to achieve 80% confluency after overnight incubation and the cells washed twice with Opti-MEM® I Reduced Serum Media (Life Technologies) and 30 μl fresh Opti-MEM added to each well at room temperature. Twenty microlitre of freshly prepared transfection mix consisting of 0.4 μg each of pSV-β-Galactosidase Control Vector (Promega) and the pGL3/MaeuCath1 promoter constructs and 1.3 μl FuGENE® HD Transfection Reagent (Promega) in Opti-MEM was added to each well. In the control wells pGL3/MaeuCath1 promoter constructs were replaced with pGL3 basic or pMAX-GFP plasmid constructs. The cells were incubated at 37°C/5% CO₂ for 5 hr and 200 μl growth media added to the transfection mix in each well and the cells left incubating for 48 hr following which the pMAX-GFP transfected wells were observed under a fluorescent microscope to determine transfection efficiency. Media was aspirated and cells washed twice with PBS making sure to completely remove residual PBS and cells lysed in Reporter Lysis Buffer (Promega) according to supplier’s instructions. Luciferase
activity was measured using the GloMax® 96 Microplate Luminometer and data normalised using β-Galactosidase activity.

Preparation of nuclear extract

Prior to Electrophoretic Mobility Shift Assays (EMSA), WallMEC from P2A were cultured in 2x T75 TC-treated culture flasks (Corning) in growth media alone (plastic) or growth media underlaid with ECM from P2A or P2B wallaby mammary tissue until they were fully confluent and nuclear extract isolated using the NE-PER kit (Pierce) according to manufacturer’s instructions. The protein concentration of the nuclear extract was estimated using the BCA Protein Assay kit (Pierce) and the extract diluted to 1 μg/μl using NER buffer (Pierce) and immediately aliquoted for storage at -80°C until required.

Electrophoretic Mobility Shift Assays (EMSA)

EMSA was performed using the Molecular Probes™ EMSA kit (E33075) according to manufacturer’s instructions. Briefly, complementary primer pairs (table 1) were annealed by mixing equimolar (100 μM) amounts in a 1.5 ml centrifuge tube and boiled in a 1l water bath for 5 min, the heater switched off and the water bath left to cool down to room temperature. Annealed primers were diluted to 10 ng/μl and 1 μl added to a binding reaction containing 4 μg nuclear extract and 2 μl binding buffer in a total volume of 10 μl and the binding mix left at room temperature for 20 minutes. On completion of the binding reaction, 2 μl of 6x DNA loading buffer was added to the mix and the complexes resolved on a 6% polyacrylamide gel in TBE at 200V. The gel was first stained with SYBR green and photographed under UV light in a ChemiDoc gel imaging system (Biorad) to view DNA bands and then stained with Sypro Ruby to view proteins.

Prediction of transcription factor binding sites
MaeuCath1 cDNA sequence [Genbank:ABV01938.1] was used to perform a BLAST search against the wallaby draft genome sequence at Ensembl and found to match the cathelicidin translated sequence on Scaffold97912. Consequently the 10 kb sequence proximal to the ATG start codon on the 5’ side was searched against the TRANFAC database for known regulatory elements including the TATA box and transcription start site, and lactation hormone responsive elements including the STAT5 binding site upon which the -2245 region was selected for further investigation using P-Match, Patch 1.0, AliBaba 2.1 software available at http://www.gene-regulation.com/pub/programs.html. Only elements that were recognised by at least two of the software at the same position in the sequence were considered. A cut-off homology of 87.5% and maximum 2 bp mismatch was allowed.

Statistical analysis

Statistical analysis of all comparative data was performed using the two-tailed t-test with a statistical significance cut-off of 5%.
4.1.7 Figures

A

Mammary gland

B

P2A WallMEC

C

P2B WallMEC

D

E

Normalized expression

plastic P2A ECM P2B ECM

NH IFPT3E2

Normalized expression

P2A WallMEC P2B WallMEC

NH IFPT3E2
Figure 1.

MaeuCath1 expression in wallaby mammary gland and mammary epithelial cells (WallMEC). **A:** Agarose gel of RT-PCR products of Maeucath1 amplified from mammary gland obtained from P2A and P2B (Wanyonyi, Sharp et al. 2011). P2A and P2B represent, phase 2A (days 2 and 80 lactation) and phase 2B (days 135, 150 and 168 lactation). Samples were loaded in order of increasing days of lactation. The splice variants MaeuCath1a and MaeuCath1b are indicated with an arrow. **B:** Agarose gel showing MaeuCath1a gene expression in P2A WallMEC cultured on ECM. PL represents plastic. P2A and P2B represent the lactation phase of the ECM. NH represents cells cultured in the absence of hormones and H represents cells treated with the full complement of lactogenic hormones (IFPT$_3$E$_2$).  **C:** Agarose gel showing MaeuCath1a gene expression in P2B WallMEC cultured on ECM. P2A and P2B represent the lactation phase of the ECM.  **D:** Quantitative PCR of MaeuCath1 using cDNA from P2A WallMEC. NH represents no hormones while IFPT$_3$E$_2$ represents the full complement of lactogenic hormones. The asterisk represents expression significantly higher than observed on P2B ECM (p $\leq$ 0.01). The & sign implies MaeuCath1a expression was significantly higher in H treated WallMEC than the NH treated WallMEC (p $\leq$ 0.04). Treatments were performed in triplicate and repeated twice. Error bars are shown.  **E:** Quantitative PCR of MaeuCath1 using cDNA from P2A and P2B WallMEC cultured on P2A ECM. The lactation phase of WallMEC is shown on the x-axis. The asterisk represents expression significantly higher in P2A WallMEC than observed in P2B WallMEC (p $\leq$ 0.002) while & implies MaeuCath1a expression was significantly higher in H treated WallMEC than the NH treated WallMEC. Treatments were performed in triplicate and repeated twice. Representative results with their error bars are shown.
Figure 2.

MaeuCath1 putative promoter sequence. Promoter sequence was obtained from the annotated wallaby genomic DNA sequence at Ensembl. Sense primers used for amplifying deletion fragments are shaded grey and shown with a forward arrow and the corresponding distance in base pairs from the ATG start site. The universal reverse primer is shown with reverse arrow. The TATA box and translation start site are shown.
Figure 3.

Deletion fragments of MaeuCath1 putative promoter and optimisation of transfection of WallMEC with reporter/promoter constructs. A: Schematic diagram of deletion fragments of the -2245 bp putative promoter. The numbers represent base pairs 5’ to the ATG translation start site. Each fragment is 14 bp shorter than the indicated size since the universal reverse primer was designed to anneal 14 bp 5’ to the start site. B: Agarose gel of promoter fragments. The sense cloning primer for each fragment was designed so as to include an Xho I restriction site while the universal reverse primer contained a Hind III restriction site. C: Optimisation of transfection of WallMEC. Cells were cultured on plastic or ECM and transfected separately with pMAX-GFP plasmid and luciferase/promoter constructs and observed under bright field and fluorescence microscope. Bright field and fluorescent images are shown.
Figure 4:

Promoter activity of the Maeucath1 putative promoter deletion fragments. A-C: Promoter activity in P2A cells cultured on P2A ECM, P2B ECM and plastic. WallMEC were cultured overnight in a 96 well plate and co-transfected with 0.8 μg each of the promoter construct, pMAX-GFP and β-galactosidase per well. Separate transfections with pGL3 basic,
pGL3 Basic/Rat beta casein (Rbeta cas on the graphs) as negative and positive controls for promoter activity respectively. Transfection with pMAX-GFP was included as a control for transfection efficiency. Luciferase activity was normalised using β-galactosidase activity. Transfections were done in triplicate and repeated three times. Representative data including error bars is shown. The asterisk represents promoter activity significantly lower than the activity of the -709 to -15 fragment (p≤0.05) D-F: Promoter activity in P2B cells cultured on P2A ECM, P2B ECM and plastic. Promoter activity higher than the activity of the -709 to -15 fragment is indicated with ** (p≤0.004).
Enhancer or repressor activity and hormone dependence of MaeuCath1 promoter.

A: Hormone response of MaeuCath1 promoter. The -709 to -15 fragment representing the highest positive regulatory effect was used to transfect P2A WallMEC cultured on P2A ECM and treated with IFPT3E2 or no hormone (NH). Luciferase activity was normalised with $\beta$-galactosidase. The asterisk represents promoter activity higher than the NH control (p=0.048).

B: Schematic diagram of deletion fragments cloned into the Rat $\beta$-casein promoter construct to test for enhancer or repressor activity. The 225 bp fragment spanning the region -709 to -485 represents a putative enhancer while the 210 bp fragment spanning -919 to -709 represents a putative repressor.

C: Activity of Rat $\beta$-casein promoter after ligating to Maeucath1 promoter fragments. The -709 to -485/R-\(\beta\)-cas and -919 to -709/R\(\beta\)-cas constructs were used to transfect P2A WallMEC cultured on P2A and treated with no...
hormone (NH) or IFPT₃E₂. Luciferase activity was normalised with β-galactosidase. Treatments were performed in triplicate and repeated twice. Representative results are shown with error bars. Promoter activity significantly lower than that of the Rat β-casein promoter is indicated by * (p=0.01) while higher activity is indicated by ** (p=0.005).
Figure 6.

MaeuCath1 promoter activity after deleting the putative repressor element. A: Schematic diagram of MaeuCath1 promoter deletion fragments without the putative...
repressor element. The repressor element is the 210 bp sequence from -919 to -710 and represented by a white box in the diagram. The predicted transcription factor binding sites are shown. The repressor-deleted fragments -2245 to -15 and -1260 to -15 are presented as a black box. The positive regulatory fragment -709 to -15 was included as a standard for activity. B: Activity of the repressor-deleted promoter fragments. Promoter constructs were used to co-transfect P2A WallMEC cultured on P2A ECM with β-galactosidase and luciferase assays performed. Luciferase activity was normalised with β-galactosidase. The asterisk represents luciferase activity significantly higher than observed for the -709 to -15 fragment (p≤0.022). Treatments were done in triplicate and repeated twice. Error bars are shown.
Figure 7.

Transcription factor binding sites on MaeuCath1 putative promoter. Transcription factor binding sites were predicted using the P-Match, Patch 1.0, AliBaba 2.1 based on the TRANSFAC® database of experimentally and in-silico determined regulatory elements. The numbers represent distance in base pairs from the ATG start site. Only elements in the forward strand and bearing higher than 87.5% homology to the experimentally determined binding site (TRANSFAC) are shown. The key of transcription factors is shown.
Figure 8: Electrophoretic mobility shift assay (EMSA) of repressor primer duplexes. Fifty base pair complementary oligonucleotides were designed spanning the 210 bp repressor fragment so as to allow 10 bp overlap between primer pairs. Nuclear extracts (equivalent to 4 μg of protein) from P2A cells cultured on plastic, P2A ECM and P2B ECM were used for binding assays. Gels were first stained with SYBR green to detect DNA and photographed and then stained with Sypro ruby to detect proteins. Both SYBR green and Sypro ruby stained images are shown. The shifted band was excised for mass spectrometry and is shown by an arrow. GS1F/GS1R, GS2F/GS2R, GS3F/GS3R, GS4F/GS4R and GS5F/GS5R represent gel shift assay primer sets in the order of increasing distance from the ATG start site. L represents DNA ladder while P, 2A and 2B represent nuclear extract isolated from cell cultured on plastic, P2A ECM and P2B ECM respectively. D represents oligonucleotide duplexes alone while D+P, D+2A and D+2B represent oligonucleotide duplexes plus nuclear extract from cells cultured on plastic, P2A ECM and P2B ECM respectively.
<table>
<thead>
<tr>
<th>Table 1.</th>
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<tr>
<td><strong>Primers for amplifying MaeuCath1 cDNA</strong></td>
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<tr>
<td>Name</td>
</tr>
<tr>
<td>MaeuCath1 Fwd</td>
</tr>
<tr>
<td>MaeuCath1 Rev</td>
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</tbody>
</table>

| **Primers for generating promoter deletions** |
| Name | Primer sequence (5’-3’) | Restriction enzyme site |
| -2245 Forward | gcaatcCTCGAGTGTTCTGGTGCTAAAAGTCTGG | Xho I |
| -1885 Forward | gcaatcCTCGAGCAAGGAAGAGATGAATATTG | Xho I |
| -1577 Forward | gcaatcCTCGAGTCACCTCAGTACAATGCGCTG | Xho I |
| -1260 Forward | gcaatcCTCGAGTGTTGGGAGTTCTTCTTTAC | Xho I |
| -919 Forward | gcaatcCTCGAGTAATCTCAGTACAATGCGCTG | Xho I |
| -709 Forward | gcaatcCTCGAGTAATCTCAGTACAATGCGCTG | Xho I |
| -484 Forward | gcaatcCTCGAGGGATTTTGGAAGGATT | Xho I |
| Common Reverse | ggatctAAGCTTATGCTCTGGGCAGAAAGAA | Hind III |

| **Primers for cloning MaeuCath1 promoter fragments into Rβ-casein promoter construct** |
| Name | Primer sequence (5’-3’) | Restriction enzyme site |
| -1260 Forward | gcaatcCTCGAGTGTTGGGAGTTCTTCTTTAC | Hind III |
| -919 Reverse | gcaatcAAGCTTATGCTCTGGGCAGAAAGAA | Hind III |
| -709 Forward | gcaatcCTCGAGTCACCTCAGTACAATGCGCTG | Xho I |
| -709 Reverse | gcaatcCTCGAGTCACCTCAGTACAATGCGCTG | Xho I |

| **Primers for gel shift assay** |
| Name | Primer sequence (5’-3’) |
| GS1F | CATATATGCACATCTCTCTGCCTCCACCTGTTTTGTCAGTGAATGAGATAGC |
| GS1R | GCTATCTCTACACTGCTTAAACAGGTGGAGAAGGATGTCTATATGT |
| GS2F | ATTGCCTACCCAAGGGATATGCTCTCTCTCTCCAGTGCATATATGAC |
| GS2R | GTGATATATGTAATGAGAAAGGAGAAGATACTTCTCTTGAGTAGAAAT |
| GS3F | GTCAATATTTGGAACACTGCTACTTTTCTCTTTCTTTCTTTCTTTTAC |
| GS3R | GGTGAAATGGAATAGAGAAAGCAGGAAATGCTCTAGCCAATTTTAC |
| GS4F | CATCTCTCTTATGTTGGGAGTGAGTCAATATAATCCATTTGGGAAAT |
| GS4R | CAAATTTGACAAATTTGGAATATATGTTGAGACTCTTACCCACATATAAGGATG |
| GS5F | TACATCTCTTGTGGTGCAAGCAAGAAGTGCAATATAATTTGACATCTCTTTAT |
| GS5R | ATAAAGGATGCAAAATTATATGCACATTTGTCTTGGACCAATTTGAAAT |

| **Primers for excluding repressor fragment from MaeuCath1 promoter (the region between -709 and -919)** |
| Name | Primer sequence (5’-3’) | Restriction enzyme site |
| -919 rep Rev | gcaatcCTCGAGCAATGGCTTGCTATTATGAG | Xho I |
| -1260 rep Fwd | gcaatcCTCGAGCAATGGCTTGCTATTATGAG | Kpn I |
| -2245 rep Fwd | gcaatcCTCGAGCAATGGCTTGCTATTATGAG | Kpn I |

Except for gel shift assay primers, all primers were designed so as to utilise restriction enzyme sites in the multiple cloning site of pGL3 basic vector and enable cloning the fragments 5’ to the luciferase or Rβ-casein gene. The respective restriction enzyme sites are shown.
Chapter Five

5.1 Transcriptome analysis (RNA-seq) of WallMEC cultured on ECM
5.1.1 Background

After confirming that the expression of milk protein genes in wallaby is regulated through temporal changes in the composition of the mammary extracellular matrix (ECM) (chapter 3), this study used the Illumina platform to perform RNA-seq in order to explore the molecular pathways which are collectively impacted by changes in ECM resulting in altered milk protein gene expression.

RNA-Seq also known as transcriptome sequencing is becoming immensely popular as a tool for gene discovery and quantitation due to a number of advantages it has over preceding technologies including DNA and RNA microarrays. Unlike microarray technologies, RNA-Seq does not require probes and hybridisation and provides the numerical frequency of individual genes thereby significantly eliminating bias and background signals (Febbo and Kantoff 2006) which characterise hybridisation technologies. Apart from generating gene expression profiles, RNA-seq enables abundant and rare transcript discovery (Su, Li et al. 2011), isoform identification (Urashima, Messer et al. 1992), elucidating alternate splice site usage (Su, Li et al. 2011) and analysis of single nucleotide polymorphisms (SNPs) (Zarnegar and Michalopoulos 1995, Zeng, Chen et al. 2002). Several platforms are available for RNA-seq including Illumina Genome Analyzer platform, ABI Solid Sequencing and 454 Sequencing provided by life sciences but all of them allow for customised sequence depth. This study utilised the Illumina genome Analyser platform.

5.1.2 Methods

WallMEC culture and isolation of RNA

Total RNA was isolated from P2A WallMECs cultured on P2B or P3 ECM in the presence of the full complement of lactogenic hormones (H) (see Chapter 3, section 4.5) or absence of
hormones (NH) and the sequencing service contracted to Beijing Genomics Institute (BGI). Culturing of WallMECs on ECM and RNA isolation was performed as described in chapter 3, section 4.5. The RNA samples were designated P2B_NH, P2B_H, P3_NH and P3_H representing WallMECs cultured on P2B ECM with no hormones, P2B ECM with hormones, P3 ECM with no hormones and P3 ECM with hormones respectively. On average 4.5 million reads, 49bp long were obtained from each sample.

**RNA-seq data analysis**

The sequence output in FastQ format (Cock, Fields et al. 2010) was screened for quality using FastQC software provided by Babraham Bioinformatics ([http://www.bioinformatics.babraham.ac.uk/projects/fastqc/](http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)) and the *Macropus eugenii* genome (Ensembl reference, Macropus_eugenii.Meug_1) used as a reference for annotation of the sequence reads. The pipeline for sequence data analysis consisted of the software described in the Tuxedo protocol (Trapnell, Roberts et al. 2012) (figure 1). Briefly sequence reads were mapped to the *M.eugenii* genome using the Bowtie2 function of TopHat 2.0.5 (Kim and Salzberg 2011, Langmead and Salzberg 2012) and transcripts assembled using Cufflinks (Trapnell, Williams et al. 2010). The final transcriptome assembly was performed by merging assembled transcripts using Cuffmerge and SAMtools (Li, Handsaker et al. 2009) and the differential expression of the mapped reads determined using Cuffdiff. In order to visualise the differential expression of transcripts, volcano plots were generated using CummeRbund (R Package) and the location where the reads aligned confirmed using SeqMonk (version 0.22.0).
Figure 1.
Functional clustering and pathway analysis

In order to perform functional annotation clustering, the gene ID’s for the differentially expressed genes (both up-regulated and down-regulated) were uploaded into DAVID v6.7 (the Database for Annotation, Visualization and Integrated Discovery) (Huang da, Sherman et al. 2009, Huang da, Sherman et al. 2009) and the gene ID’s converted to symbols which are compatible with the DAVID database. Only gene clusters with an enrichment value equal to or higher than 1.3 (following authors’ recommendations) (Huang da, Sherman et al. 2009) were considered representative of the treatments unless the cluster or pathway has previously been associated with interactions between the extracellular matrix and epithelial cells and milk protein gene expression. Pathway analysis was performed using the BioCarta and KEGG databases which are available at DAVID (http://david.abcc.ncifcrf.gov/).

5.1.3 Results and discussion

Transcript abundance

Of the 31313 transcripts available in the wallaby genome, 24582, 24646, 24899, 24804 mapped to transcripts from the P2B-NH, P2B-H, P3-NH, P3-H treatments respectively.

Differential regulation of gene expression by ECM and hormone treatments

In order to visualise the differential expression of genes between treatments, volcano plots were constructed for four datasets representing, P2B ECM with and without hormones (P2B_NH versus P2B_H), P3 ECM with and without hormones (P3_NH versus P3_H), P2B ECM and P3ECM without hormones (P2B_NH versus P3_NH) and P2B ECM and P3 ECM with hormones (P2B_H versus P3_H). The plots are shown in figures 2A – D. The dots on the left side of zero represent down regulated genes while the dots on the right side represent up-regulated genes. The statistical significance of the difference between treatments
expressed as $-\log_{10}(p\ value)$ is shown on the y-axis. Blue dots represent genes whose expression level is significantly different. In order to enhance the level of confidence only genes with $-\log_{10}(p\ value)$ higher than 2.5 were differentially expressed between the treatments.

Figure 2A

Volcano plot comparing the fold change in gene expression of WallMEC cultured on P2B ECM without hormones (NH) and in the presence of hormones (H). Except for one
unannotated gene there were no significantly differentially expressed genes between H and NH treated WallMEC.

Figure 2B

Volcano plot comparing the fold change in gene expression of WallMEC cultured on P3 ECM without hormones (NH) and in the presence of hormones (H). Fourteen genes were differentially regulated (13 up-regulated and 1 down-regulated in the presence of hormones). Five of the up-regulated genes were identified as CSF3, ENSMEUG00000006814, MMP1,
OLFM4 and GPR83. The remaining 7 genes were unknown. The down-regulated gene was unknown.
Volcano plot comparing the fold change in gene expression of WallMEC cultured on P2B ECM versus P3 ECM without hormones (H). Relative to P2B ECM, 598 genes were up-regulated by P3 ECM (303 annotated or wallaby transcript ID known, 295 unknown) and 203 down-regulated (75 annotated or wallaby transcript ID known, 128 unknown).
Figure 2D

Volcano plot comparing the fold change in gene expression of WallMEC cultured on P2B ECM versus P3 ECM with hormones (H). Relative to P2B ECM 601 genes were up-regulated (286 annotated or wallaby transcript ID known, 315 unknown) and 207 genes were down-regulated (79 annotated or wallaby transcript ID known, 128 unknown).
### Table 1

Summary of differentially regulated genes after culturing WallMEC on P2B or P3 ECM in the presence or absence of lactogenic hormones. Annotated represents genes whose ID is available at GenBank and/or Ensembl and genes which have no gene name but have been assigned a transcript number at Ensembl eg ENSMEUG00000006814. Unknown represents genes that returned no gene or transcript ID when mapped to the wallaby genome.

<table>
<thead>
<tr>
<th>Treatments compared</th>
<th>Up-regulated</th>
<th></th>
<th></th>
<th>Down-regulated</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Annotated</td>
<td>Unknown</td>
<td>Total</td>
<td>Annotated</td>
<td>Unknown</td>
<td>Total</td>
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<tr>
<td>P2B_NH versus P2B_H</td>
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<td>1</td>
<td>1</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>P3_NH versus P3_H</td>
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<td>7</td>
<td>13</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>P2_NH versus P3_NH</td>
<td>303</td>
<td>295</td>
<td>598</td>
<td>75</td>
<td>128</td>
<td>203</td>
</tr>
<tr>
<td>P2_H versus P3_H</td>
<td>286</td>
<td>315</td>
<td>601</td>
<td>79</td>
<td>128</td>
<td>207</td>
</tr>
</tbody>
</table>

*Functional clusters of differentially expressed genes*
In order to determine the differential effect of ECM and hormones on WallMEC gene expression, functional clusters of genes were generated by uploading the ID’s of differentially regulated genes from the P2B_NH versus P2B_H, P3_NH versus P3_H, P2_NH versus P3_NH and P2_H versus P3_H treatments onto the DAVID functional annotation software. The relative abundance of functional clusters for each dataset are shown in figures 3A-D. No clustering was done for the P2B_NH versus P2B_H because only one gene was differentially expressed. Although functional annotation was attempted for the 5 differentially expressed genes in the P3_NH versus P3_H dataset, no functional cluster was obtained.

In all the datasets glycoproteins constituted the highest percentage of differentially expressed genes, averaging 16% (figure 3A-D). Post-translational glycosylation of proteins regulates the function of a large number of proteins and in the context of milk, glycosylation protects proteins from proteolysis (Brines and Brock 1983), modulates interaction of milk antimicrobials with target pathogens (Newburg 1999) and plays a role in neonatal development. Therefore the differential expression of a large number of glycoprotein genes upon culturing WallMEC on ECM points to a global rather than specific modulatory role of ECM in MEC function.

Also prominent in all the datasets were the ECM and cell adhesion clusters (3%), dominated by matrix metalloproteases (MMP’s) and ADAM metallopeptidase with thrombospondin (ADAMTS’s) and they appeared to be regulated both by the ECM and hormones. For example while MMP1, MMP7, MMP9, MMP16, ADAMTS1, ADAMTS8 and ADAMTS15 were up-regulated in the P2B_H versus P3_H dataset, only MMP1, MMP16 and ADAMTS15 were up-regulated in the P2_NH versus P3_NH dataset (higher in P3_NH; tables 3 and 4). The ECM related cluster also consisted of structural ECM proteins including collagen VII, a basal lamina protein secreted by epithelial cells and collagen XV which is
expressed mainly in fibroblasts but also in renal, pancreatic, lung and placental epithelial cells (Kivirikko, Saarela et al. 1995). Tenascin C which is expressed in fibroblasts but also in mammary epithelial cells (Gonzalez-Sancho, Alvarez-Dolado et al. 1999) in a lactation phase-dependent manner (Jones, Boudreau et al. 1995, Wirl, Hermann et al. 1995) was also differentially regulated by the ECM (tables 3 and 4).

Mammary luminal epithelial cells have been shown to express various MMP’s (Djonov, Hogger et al. 2001, Park, Zeng et al. 2001) and ADAMTS’s (Porter, Scott et al. 2004) constitutively and in an inducible manner although these proteases are mainly expressed in the stroma and myoepithelium (Porter, Scott et al. 2004). Therefore it is conceivable that fibroblasts were present in the WallMEC culture and this study should have included epithelial-enriching and fibroblast-sequestering steps. However the overarching cause for change in WallMEC phenotype was evidently the synergistic effect of hormones and the ECM since there were marked differences in gene expression between P2B ECM and P3 ECM treated cells.

Similar to the ECM related cluster, genes in the hormone response cluster were abundant (3%) in both NH and H datasets (figures 3A-D) but surprisingly absent in the down-regulated gene category of both clusters (tables 5 and 6). This lop-sided expression of hormone response genes requires further investigation because it can help dissect the hormone effect from the ECM effect.
**Figure 3A:** Relative abundance of up-regulated functional clusters for the P2_NH versus P3_NH dataset.
Figure 3B

Relative abundance of down-regulated functional clusters for the P2_NH versus P3_NH dataset.
Figure 3C: Relative abundance of up-regulated functional clusters for the P2_H versus P3_H dataset.
Figure 3D
Relative abundance of up-regulated functional clusters for the P2_H versus P3_H dataset.
<table>
<thead>
<tr>
<th>Up-regulated genes (P2B_NH &lt; P3_NH)</th>
<th>DAVID Enrichment score</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAM metallopeptidase with thrombospondin type 1 motif, 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD248 molecule, endosialin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kazal-type serine peptidase inhibitor domain 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>acetylcholinesterase (Yt blood group)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>amyloid beta (A4) precursor-like protein 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>angiogenin, ribonuclease, RNase A family, 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bone morphogenetic protein 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cartilage oligomeric matrix protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>coagulation factor III (thromboplastin, tissue factor)</td>
<td>3.76</td>
<td>6.0E-6</td>
</tr>
<tr>
<td>collagen, type VII, alpha 1</td>
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<td></td>
</tr>
<tr>
<td>collagen, type XV, alpha 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fibulin 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lysyl oxidase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>matrix metallopeptidase 1 (interstitial collagenase)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>matrix metallopeptidase 16 (membrane-inserted)</td>
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<td></td>
</tr>
<tr>
<td>netrin 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tenascin C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tumor necrosis factor receptor superfamily, member 11b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wingless-type MMTV integration site family, member 7A</td>
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<td></td>
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</table>

<table>
<thead>
<tr>
<th>Down-regulated genes (P2B_NH &gt; P3_NH)</th>
<th>DAVID Enrichment score</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAM domain containing 2</td>
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<td></td>
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<tr>
<td>SPARC related modular calcium binding 2</td>
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<td></td>
</tr>
<tr>
<td>cell adhesion molecule with homology to L1CAM (close homolog of L1)</td>
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<tr>
<td>collagen, type XIV, alpha 1</td>
<td>2.59</td>
<td>1.6E-4</td>
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<tr>
<td>matrix metallopeptidase 19</td>
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<td></td>
</tr>
<tr>
<td>matrix metallopeptidase 7 (matrilysin, uterine)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>spondin 2, extracellular matrix protein</td>
<td></td>
<td></td>
</tr>
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Table 3

Differentially regulated extracellular-matrix related genes in WallMEC cultured on P2B or P3 ECM without hormones (P2B_NH versus P3_NH). The genes marked in bold letters are unique to the NH treated samples.
<table>
<thead>
<tr>
<th>Up-regulated genes (P2B_H &lt; P3_H)</th>
<th>DAVID Enrichment score</th>
<th>P value</th>
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<tbody>
<tr>
<td>ADAM metallopeptidase with thrombospondin type 1 motif, 1</td>
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<td>ADAM metallopeptidase with thrombospondin type 1 motif, 15</td>
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</tr>
<tr>
<td>Kazal-type serine peptidase inhibitor domain 1</td>
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<td></td>
</tr>
<tr>
<td>amyloid beta (A4) precursor-like protein 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bone morphogenetic protein 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cartilage oligomeric matrix protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chitinase 3-like 1 (cartilage glycoprotein-39)</td>
<td></td>
<td></td>
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<tr>
<td>coagulation factor III (thromboplastin, tissue factor)</td>
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<td></td>
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<tr>
<td>collagen, type XV, alpha 1</td>
<td></td>
<td></td>
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<tr>
<td>fibulin 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>matrix metallopeptidase 1 (interstitial collagenase)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>matrix metallopeptidase 16 (membrane-inserted)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>matrix metallopeptidase 7 (matrilysin, uterine)</td>
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<td></td>
</tr>
<tr>
<td>matrix metallopeptidase 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tenascin C</td>
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<td></td>
</tr>
<tr>
<td>tumor necrosis factor receptor superfamily, member 11b</td>
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<td></td>
</tr>
<tr>
<td>wingless-type MMTV integration site family, member 7A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Down-regulated genes (P2B_H &gt; P3_H)</td>
<td>DAVID Enrichment score</td>
<td>P value</td>
</tr>
<tr>
<td>ADAM metallopeptidase with thrombospondin type 1 motif, 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAM domain containing 2</td>
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</tr>
<tr>
<td>SPARC related modular calcium binding 2</td>
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</tr>
<tr>
<td>cell adhesion molecule with homology to L1CAM (close homolog of L1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>extracellular matrix protein 2, female organ and adipocyte specific</td>
<td></td>
<td></td>
</tr>
<tr>
<td>matrix metallopeptidase 19</td>
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<td></td>
</tr>
</tbody>
</table>

Table 4

Differentially regulated extracellular-matrix related genes in WallMEC cultured on P2B or P3 ECM in the presence of hormones (P2B_H versus P3_H). The genes marked in bold letters are unique to the H treated samples.
### Table 5

Differentially regulated hormone response related genes in WallMEC cultured on P2B or P3 ECM without hormones (P2B_NH versus P3_NH)

<table>
<thead>
<tr>
<th>Up-regulated genes</th>
<th>DAVID Enrichment score</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>adrenomedullin</td>
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<td>angiogenin, ribonuclease, RNase A family, 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bone morphogenetic protein 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>crystallin, alpha B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cytochrome P450, family 27, subfamily B, polypeptide 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>enolase 2 (gamma, neuronal)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gamma-glutamyl hydrolase (conjugase, folypolygammaglutamyl hydrolase)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>growth hormone receptor</td>
<td>2.16</td>
<td>6.5E-4</td>
</tr>
<tr>
<td>high-mobility group box 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>insulin-like growth factor 2 (somatomedin A); insulin; INS-IGF2 readthrough transcript</td>
<td></td>
<td></td>
</tr>
<tr>
<td>interleukin 6 (interferon, beta 2)</td>
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<td></td>
</tr>
<tr>
<td>lysyl oxidase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nuclear receptor subfamily 4, group A, member 3</td>
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<td></td>
</tr>
<tr>
<td>phospholipase A2, group IVA (cytosolic, calcium-dependent)</td>
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<td></td>
</tr>
<tr>
<td>tumor necrosis factor receptor superfamily, member 11b</td>
<td></td>
<td></td>
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<tr>
<td>very low density lipoprotein receptor</td>
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<td></td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Down-regulated genes</th>
<th>Enrichment score</th>
<th>p-Value</th>
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<tbody>
<tr>
<td>No functional cluster identified</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 6
Differentially regulated hormone response related genes in WallMEC cultured on P2B or P3 ECM in the presence of hormones
Differentially regulated functional pathways

Because only one gene (unknown) was differentially expressed between no hormone (NH) and hormone-treated (H) WallMEC cultured on P2B ECM, pathway analysis was not done for the P2B-NH versus P2B_H dataset. Similarly, although pathway analysis was done for the 5 differentially expressed genes in the P3_NH versus P3_H dataset, the query did not return any pathway. The comparison between WallMEC cultured on P2B ECM and P3 ECM in the presence of hormones (P2B_H versus P3_H) identified 12 pathways in which genes were differentially up-regulated (higher in P3_H; table 7). Nine out of these pathways were affected in the comparison between P2B ECM and P3 ECM in the absence of hormones (P2B_NH versus P3_NH). Therefore although there appeared to be little or no influence of hormones in the change of WallMEC phenotype (table 1), the hormones appeared to exert their effect in the presence of ECM. For example, while the entire p53 signalling pathway was not affected in the P2B-NH versus P3_NH dataset, five genes in the pathway were up-regulated in the P2B-H versus P3_H dataset (higher in P3_H; table 7). Similarly, although the Galactose metabolism and Nod-like receptor signalling pathways were up-regulated in the P2B_NH versus P3_NH dataset (higher in P3_NH), their enrichment score was significantly lower than the P2B_H versus P3_H dataset and below the cut-off of 1.3.

Equally interesting, three down-stream member genes of the canonical Wnt signaling pathway including Fra1, CyclinD and Uterine which link Wnt signaling to cell cycle and Smad3 which links it to TGF-β signaling were only up-regulated in the presence of hormones (table 7). The effect of hormones on WallMEC phenotype is further confirmed by the down-regulation of genes in three functional clusters including the lysosome pathway, complement and coagulation pathway and cell adhesion molecules (table 7B) only in the presence of hormones. These observations confirm a key hypothesis in this study that the ECM sensitizes mammary epithelial cells to lactogenic hormones.
### Table 7

#### Up-regulated

<table>
<thead>
<tr>
<th>P2B_ NH versus P3_ NH</th>
<th>Regulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jak-STAT signaling pathway</td>
<td>IL6, CytokineR, PI3K, CycD</td>
</tr>
<tr>
<td>Cytokine-cytokine receptor interaction</td>
<td>IL6, IL11, LIF, BSF3, CSF3, GHR, CSF2RB, SF11B, SF21, IL1R2, HGF, FLT, IL18</td>
</tr>
<tr>
<td>Complement and coagulation cascades</td>
<td>F3, THBD, serpinE1</td>
</tr>
<tr>
<td>Hematopoietic cell lineage</td>
<td>G-SCF, IL-6, IL-11,CD121</td>
</tr>
<tr>
<td>TGF-beta signaling pathway</td>
<td>THBS1, BMP, FST, Smad1/5/8</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>Bub1, BubR1, cd20, cyclinB, Plk1, cyclinD, MCM5</td>
</tr>
<tr>
<td>oocyte meiosis</td>
<td>IGF-1, Bub1, PI3K, cdc20, Plk1, CycB1, AC</td>
</tr>
<tr>
<td>Neurotrophin signaling pathway</td>
<td>TrkC, PI3K, IkB, PKCδ, RIP2, IRAK</td>
</tr>
<tr>
<td>Wnt signaling pathway</td>
<td>FRP, Wnt, Frizzled, Nkd, NFAT</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P2B_H versus P3_H</th>
<th>Regulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jak-STAT signaling pathway</td>
<td>IL6, CytokineR, PI3K, Cycdin</td>
</tr>
<tr>
<td>Cytokine-cytokine receptor interaction</td>
<td>IL6, IL11, LIF, BSF3, CSF3, GHR, CSF2RB, SF11B, SF21, IL1R2</td>
</tr>
<tr>
<td>Complement and coagulation cascades</td>
<td>F3, THBD, serpinE1, PLAU, DF</td>
</tr>
<tr>
<td>Hematopoietic cell lineage</td>
<td>G-SCF, IL-6, IL-11,CD121</td>
</tr>
<tr>
<td>TGF-beta signaling pathway</td>
<td>Smad2,3, THBS1, BMP, FST, Smad1/5/8</td>
</tr>
<tr>
<td>p53 signaling pathway</td>
<td>CyclinD, CyclinB, IGF, PAI, p53R2</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>Bub1, BubR1, Smad2,3, cd20, cyclinB, Plk1, cyclinD,</td>
</tr>
<tr>
<td>Oocyte meiosis</td>
<td>IGF-1, Bub1, PI3K, cdc20, Plk1, CycB1, AC</td>
</tr>
<tr>
<td>Neurotrophin signaling pathway</td>
<td>TrkC, PI3K, IkB</td>
</tr>
<tr>
<td>NOD-like receptor signaling pathway</td>
<td>RIP2, IkB, IL-6</td>
</tr>
<tr>
<td>Wnt signaling pathway</td>
<td>FRP, Wnt, Frizzled, Nkd, Smad3, NFAT, fra1, CycD, uterine</td>
</tr>
<tr>
<td>Galactose metabolism</td>
<td>EC 2.7.1.1, EC 2.7.7.9, EC 5.1.3.2, EC 5.4.2.2</td>
</tr>
</tbody>
</table>

### Table 7B

#### Down-regulated

<table>
<thead>
<tr>
<th>P2B_ NH versus P3_ NH</th>
<th>Regulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pathway</td>
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</table>

<table>
<thead>
<tr>
<th>P2B_H versus P3_H</th>
<th>Regulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysosome</td>
<td>cathepsins,ACP5, ASAH1, LAPTM</td>
</tr>
<tr>
<td>Complement and coagulation pathway</td>
<td>C1S, C2, C3,</td>
</tr>
<tr>
<td>Cell adhesion molecules</td>
<td>PVRL1, CNTN1, CDH3,</td>
</tr>
</tbody>
</table>

Red = Differentially up-regulated between H and NH. Green = Enrichment score of P2B_ NH versus P3_ NH significantly below cut-off. Blue = Pathway missing in the NH treated datasets.
p53 signaling

Four members of the p53 signaling pathway including Cyclin B, Cyclin D, IGF (insulin-like growth factor) and p53 R2 (the p53-regulated R2 subunit of ribonucleotide reductase) were up-regulated in P3_H treated WallMEC relative to the P2B_H (table 7A; figure 4A). The p53 protein is a well known tumor suppressor that is induced by stress signals including hypoxia, nutrition deprivation and DNA damage agents (Oren 1999, Ryan, Phillips et al. 2001). p53 transcriptionally regulates several genes including p21, Reprimo and Gadd45 which repress cyclins leading to cell cycle arrest (Levine, Hu et al. 2006). The p53 signalling pathway also includes the activation of caspases (leading to apoptosis) (Haupt, Berger et al. 2003) and regulation of DNA repair through activation of p48 (Levine, Hu et al. 2006).

Cyclins B and D belong to the cyclin family of proteins which positively regulate the progression of cells through the cell cycle (Nigg 1995) and therefore their relative up-regulation by treating cells with P3-H (P3 ECM in the presence of hormones) implies that P3 ECM promotes cell division. This is consistent with the significant increase in the size of the mammary gland during phase 3 of wallaby lactation (Bird, Hendry et al. 1994). Similarly the increased expression of IGF implies that P3 ECM promotes the proliferative effect of growth hormone (GH) since GH exerts its growth stimulus through the induction of IGF expression.

However the up-regulation of p53R2 was somewhat discordant with the proliferative function of P3 ECM since p53R2 has been shown to arrest cell cycle by suppressing cyclin D1 expression (Zhang, Wu et al. 2011). In the context of wallaby lactation, the balance between the proliferative activity of cyclins and the cell-cycle-arresting activity of p53R2 may be needed to enable enhanced mammary growth during P3 while allowing for gradual involution which is evident in late lactation (Khalil, Digby et al. 2008).
**Figure 4A:**

**p53 signaling pathway.** Up-regulated genes are marked with a red star.
Galactose metabolism

At the onset of phase 3 of wallaby lactation, there is a dramatic decrease in the carbohydrate content of milk that has been attributed to increased expression of β-galactosidase (Green, Newgrain et al. 1980, Green, Griffiths et al. 1983) and UDP-galactose hydrolase which catalyses the breakdown of UDP-galactose, a constituent monosaccharide of lactose (Messer and Nicholas 1991). Because changes in enzymes that catalyse galactose metabolism (β-1,3 galactosyltransferase and β-1,4 galactosyltransferase) are dependent on the phase of lactation in the wallaby, it has been suggested that these enzymes are regulated by the endocrine system and unknown factors within the mammary gland (Menzies and Nicholas 2007). This study reports for the first time the increase in the expression of the genes for four enzymes (EC 2.7.1.1, EC 2.7.7.9, EC 5.1.3.2 and EC 5.4.2.2) involved in galactose metabolism after culturing WallMEC on P3 ECM relative to P2B ECM (table 7; figure 4B).

UDP-glucose 6-dehydrogenase (EC 2.7.7.9) catalyses the reversible conversion of UDP-glucose into α-D-Glucose-1-P while glucose phosphomutase (EC 5.4.2.2) catalyses the conversion of α-D-Glucose-1-P to α-D-Glucose-6P both of which are downstream steps in the incorporation of D-galactose into the glycolysis pathway (Berg JM, Tymoczko JL et al. 2002). UDP-galactose 4-epimerase (EC 5.1.3.2) reversibly converts UDP-galactose to UDP-glucose (Holden, Rayment et al. 2003) while hexokinase (EC 2.7.1.1) phosphorylates α-D-Glucose to form α-D-Glucose-6P (Berg JM, Tymoczko JL et al. 2002). This data suggests that although the reactions catalysed by these enzymes are reversible, changes in the ECM may progressively tilt the equilibrium towards catabolism of carbohydrates leading to the marked decrease in carbohydrates during P3.
Figure 4B: Galactose metabolism pathway. Up-regulated genes are marked with a red star.
**TGF-β signalling**

Three genes in the TGF-β (Transforming growth factor beta) signalling pathway, including BMP, Smad8, THBS1 were up-regulated in the P2B-NH versus P3_NH and P2B-H versus P3_H datasets (higher in P3) while Smad3 was only up-regulated in the P2B-H versus P3_H dataset. TGF-β negatively regulates epithelial cell proliferation (Moustakas, Pardali et al. 2002) and has been known to induce apoptosis in several cells through the Smad pathway (Jang, Chen et al. 2002). TGF-β exists in a latent form as a TGF-β complex and is activated through proteolytic cleavage by matrix metalloproteases 2 and 9 in an integrin-dependent manner (Yu and Stamenkovic 2000). Studies have shown that TGF-β is down-regulated when MEC are cultured on ECM (Streuli, Schmidhauser et al. 1993) and it represses β-casein expression in a Smad-dependent manner in MEC (Wu, Lee et al. 2008) implying that TGF-β may inhibit MEC differentiation. The relative up-regulation of genes in the TGF-β signaling pathway by P3 ECM is therefore worth further investigation.
Figure 4C: TGF-β signalling. Up-regulated genes are marked with a red star.
**Wnt Signalling pathway**

Nine genes in the canonical Wnt signaling pathway including the Wnt receptor, Frizzled and FRP (Frizzled related protein), an inhibitor of the Wnt/β-catenin signaling pathway were up-regulated in the P2B-H versus P3_H dataset (higher in P3_H). Five of these genes including FRP, Wnt, Frizzled, Nkd and NFAT were also up-regulated in the P2B_NH versus P3_NH dataset (higher in P3_NH). Notably, three genes, Fra1, CycD and Uterine which link the Wnt signaling pathway to cell cycle progression (Gordon and Nusse 2006) were only up-regulated in the hormone treated samples. Similarly, Smad3 which links Wnt signaling to TGF-β signaling pathway (Gordon and Nusse 2006, McNeill and Woodgett 2010) was expressed exclusively in the hormone treated samples.

The Wnt signaling pathway is involved in several cellular process including cell proliferation (Logan and Nusse 2004), stem cell differentiation (Nusse 2008), gastrulation (Biechele, Cox et al. 2011), anti-apoptotic protection of several cell types (Chen, Guttridge et al. 2001, Shin, Park et al. 2009, Fragoso, Yi et al. 2011) and stimulation of angiogenesis through positive regulation of the Vascular Endothelial Growth Factor (VEGF)(Zerlin, Julius et al. 2008, Phng, Potente et al. 2009). Therefore it is possible that the balance between the activation of Wnt signaling and FRP-mediated inhibition of Wnt signaling is regulated by the synergistic effect of the ECM and the hormones in order to stimulate vascularisation during P3 while allowing gradual involution (Chapter 3, section 3).
Figure 4D: Wnt Signalling pathway. Up-regulated genes are marked with a red star.
JAK-STAT Signaling

The JAK/STAT system is a pleiotrophic signalling pathway that is activated by a number of ligands including erythropoietin (EPO), growth hormone (GH), interleukins (Niemand, Nimmesgern et al. 2003) and interferons (Lau and Horvath 2002) leading to several cellular outcomes including cell proliferation, differentiation (Nishikomori, Usui et al. 2002) and migration. In mammals, major physiological processes including, mammary gland development, hematopoiesis, sexual differentiation and adipogenesis are regulated by the JAK/STAT signalling cascade (O'Shea, Gadina et al. 2002) (Aaronson and Horvath 2002, Lau and Horvath 2002). It is also evident that the response of the mammary gland to prolactin, leading to milk protein gene expression and lactation is mediated by the JAK/STAT signalling pathway through Jak2 and Stat5 (Watson and Burdon 1996).

In this study four members of the JAK/STAT signalling cascade including IL-6, CytokineR PIK3 and CycD were up-regulated in both the P2B_NH versus P3_NH and P2B_H versus P3_H datasets (higher in P3). Therefore it appears that the differential expression of these signaling molecules was not hormone-dependent and is likely to be mediated by the ECM. This is consistent with earlier studies in the wallaby which showed that although there is increased prolactin expression during P3 (Nicholas 1988), the change in milk protein gene expression pattern between P2B and P3 was not dependent on hormones (Nicholas, Simpson et al. 1997). Further, PI3K and CycD, the two signalling proteins downstream of JAK (figure 4F) which were up-regulated in this study are likely to induce apoptosis (through PI3/Akt signaling) (Morgensztern and McLeod 2005) and regulate cell division (Nigg 1995) respectively rather than induce milk protein expression.
Figure 4E: JAK-STAT Signaling. Up-regulated genes are marked with a red star.
The lysosome functional cluster was among the three differentially down-regulated pathways in the P2B_H versus P3_H dataset (lower in P3_H), with four genes including cathepsin S, ACP 5 (Acid phosphatase 5), ASAH1 (N-acylsphingosine amidohydrolase) and LAPT M (Lysosomal-associated transmembrane protein 4B) being down-regulated (table 7B). Cathepsin S is a cysteine protease that is constitutively expressed in immune cells (Shi, Villadangos et al. 1999) and is thought to signal apoptosis through the cleavage of Bid and the consequent release of cytochrome C from mitochondria (Stoka, Turk et al. 2001, Watson and Kreuzaler 2009). Cathepsins that belong to the cysteine protease family have been associated with the necrotic cell death through lysosomal membrane permeabilisation (LMP) during mammary involution (Ferri and Kroemer 2001, Boya and Kroemer 2008). ACP5 catalyses the release of mannose 6-phosphate the lysosome-targeting label from proteins which are marked for proteolytic degradation in the lysosome (Sun, Sleat et al. 2008). ASAH1 (N-acylsphingosine amidohydrolase) also known as acid ceramidase digests a type of spingolipids called ceramides and has been shown to regulate steroidogenesis and adrenocortical function (Lucki, Bandyopadhyay et al. 2012). The down-regulation of lysosomal proteases, manose 6-phosphatase and ceramidases upon culturing WallMEC on P3 ECM could imply decreased proteolysis and lipid catabolism which is consistent with high protein and lipid content in milk during P3 of wallaby lactation (Nicholas 1988, Nicholas, Simpson et al. 1997, Kwek, Wijesundera et al. 2007). In bovine milk ceramides represent close to 30% of total phospholipid (Parodi 1997) and it would interesting to investigate ceramide metabolism in wallaby.
The complement and coagulation cascade

The complement system is a multi-tasking family of more than 30 proteins that function in a cascade to effect immunological reaction to pathogens by opsonising pathogens, activation of immune cells, chemotaxis, and clearance of immune cells among other functions (Brown 1991, Brown 1992). In this study two of the five up-regulated genes (PLAU and DF) in the complement and coagulation system were up-regulated only in the P2B_H versus P3_H dataset (table 7A). PLAU (Urokinase-type plasminogen activator) is a serine protease that plays a role in the breakdown of the ECM by cleaving plasminogen to form plasmin. PLAU therefore may provide a link between a possible hormone-ECM mediated remodelling of the extracellular matrix during P3 of wallaby lactation and is worth further investigation. DF (Complement factor D) plays a role in in the alternative complement pathway by cleaving Bf (Zhou, Liu et al. 2012)

Of particular interest is the down-regulation of the three serially connected members (C1s, C2 and C3) of the classical complement pathway in the P2B_H versus P3_H dataset (table 7B). The mu chain of immunoglobulin M (IgM) has recognition sites for complement 1q (C1q) which upon binding to IgM activates C1s and C1s proteolitically cleaves C2 and C4 into two fragments each (Walport 2001, Walport 2001). The larger fragments of C2 and C4 (C2b and C4b respectively) then heterodimerise to form C4b.C2b which cleaves C3 to release C3b a well known opsonin for macrophages and neutrophils (Walport 2001, Walport 2001, Wallis, Mitchell et al. 2010). Whether this down-regulation of the complement molecules is aimed at preventing autophagy of immune cells during P3 is not clear from this study. However it is apparent that the ECM and hormones act synergistically to regulate immune activity during P3 through the complement cascade.
Other signalling pathways

Among the differentially regulated signalling systems in both the P2B_NH versus P3_NH and P2B_H versus P3_H datasets were the cytokine-cytokine receptor interaction (table 7A; appendix 6), hematopoietic cell lineage, cell cycle, oocyte meiosis and the neurotrophin signaling pathway (table 7A). Thirteen genes (IL6, IL11, LIF, BSF3, CSF3, GHR, CSF2RB, SF11B, SF21, IL1R2, HGF, FLT, IL18) in the cytokine-cytokine receptor interaction cluster were up-regulated in the P2B_NH versus P3_NH dataset (higher in P3_NH). Out of the thirteen, three (HGF, FLT, IL18) were differentially expressed (higher in the NH than the H datasets) implying that their expression may be repressed by hormones in an ECM dependent manner.

Four genes (TrkC, PI3K, IkB, IRAK) in the neurotrophin signaling pathway were up-regulated in both the P2B_NH versus P3_NH and P2B_H versus P3_H datasets (higher in P3). However PKCδ, another member of the neurotrophin pathway was up-regulated only in the NH dataset while RIP2 was only up-regulated in the H dataset. Similarly in the cell cycle cluster, MCM5 was up-regulated in the NH dataset while Smad3 was up-regulated in the H dataset, implying that the expression of these genes is regulated by lactogenic hormones in an ECM dependent manner.

Both the H and NH datasets shared the up-regulation of the hematopoietic cell lineage genes G-SCF, IL-6, IL-11 and CD121 suggesting that the expression of these genes is dependent on the ECM rather than hormones. Similarly, the oocyte meiosis cluster (AC, IGF-1, Bub1, PI3K, cdc20, Plk1, CycB1) was up-regulated in both the NH and H datasets (P3 ECM) implying that their expression is dependent on the ECM.
Milk protein gene expression

Beta-casein, the control for induction of lactation in this study was represented by 7 transcripts the most abundant of which had FPKM values of 1939, 2652, 1416 and 1522 for the P2B_NH, P2B_H, P3_NH and P3_H treatments respectively (figure 5A). These FPKM values placed β-casein among the top 2.4%, 2%, 3.4% and 3.3% most abundantly expressed transcripts in the respective treatments. Similarly β-lactoglobulin had FPKM values of 626, 786, 282 and 664 placing it among the top 4.9%, 4.3%, 8.1% and 5.0% most abundantly expressed transcripts respectively (figure 5B). However the difference between the H and NH treatments for β-casein was smaller than the expected 2-3 fold difference observed using quantitative PCR on RNA isolated from similarly treated WallMEC (chapter 3, section 2.4). This discrepancy may have arisen from a number of sources including the difference in sensitivity between RNA-seq and PCR due to positional bias by RNA-seq (Roberts, Trapnell et al. 2011) and the ability of the data analysis pipeline to detect the relative quantities of individual transcripts of a gene. The cufflinks suite used for transcript assembly in this study relies on the accurate mapping of every read to its source isoform (Trapnell, Williams et al. 2010, Trapnell, Roberts et al. 2012) and may function sub-optimally with genomes that are not fully annotated (Trapnell, Williams et al. 2010) as is the case with the wallaby genome. This may also be the reason why no tWAP transcript was identified when the reads were mapped to the wallaby genome. Taken together β-casein was expressed at significantly higher levels than the bulk of the genes in the WallMEC transcriptome suggesting lactation was induced.

The FPKM values for the early lactation protein (tELP) were less than 25 for all treatments although P3 ECM appeared to elevate its expression level (figure 5C). The expression of tELP by P2A WallMEC on P2B and P3 ECM is likely to have resulted from P2A WallMEC retaining their phenotype and constitutively expressing tELP (Nicholas, Simpson et al. 1997,
Simpson, Bird et al. 1998) rather than an ECM effect. RT-PCR data (chapter 3, section 2.4 and section 6, figure 4) showed that tELP was neither responsive to hormones or ECM and the difference observed presently may be an artefact arising from low FPKM values.

Late lactation protein A ( LLP-A) was represented by two transcripts. The more abundant transcript had FPKM of 108, 82, 66 and 61 values for the P2B_NH, P2B_H, P3_NH and P3_H treatments respectively, placing it in the top 13% most abundantly expressed transcripts in the P2B_NH treatment category. Using single radial immunodiffusion, early studies (Nicholas, Messer et al. 1987) showed that LLP-A is detectable in wallaby milk after week 24 post-partum. Consistent with data reported in chapter 3, Section 2.4 and figure 6 in which P2B ECM changed the phenotype of P2A cells to adopt a P2B phenotype, it is conceivable that P2B ECM (day 168 lactation) used in this study changed the phenotype of P2A WallMEC to express tLLP-A.

Similar to tELP, the expression of tLLP-B was relatively low (the more abundant of two transcripts having the highest FPKM of 39.9) and the expression was neither hormone nor ECM dependent. This data is consistent with qPCR data in which both P2B and P3 ECM did not induce tLLP-B expression in P2B WallMEC leading to the conclusion that although latter phase ECM changes WallMEC phenotype to the latter phase, it does not fast-track the phenotype by more than one lactation phase.

MaeuCath1 was represented by one transcript and its expression was the most ECM-dependent among the milk protein genes. While the FPKMs for P2B-NH and P2B-H treatments were 24.7 and 45.4 respectively, the FPKMs for P3-NH and P3-H were 1487.7 and 1075.3 respectively. MaeuCath1 has two splice variants, MaeCath1a and MaeuCath1b (Wanyonyi, Sharp et al. 2011) and WallMEC only express MaeuCath1a (chapter 4, section 2.1). The significant down-regulation of MaeuCath1a by P2B ECM is consistent with the

Collectively this study has shown that the ECM and lactogenic hormones complement each other to change lactation phenotype from one phase of wallaby lactation to the other through the regulation of multiple molecular pathways.
Figure 5: Milk protein gene expression level in FPKM

On the y-axis is the gene expression level in fragments per kilobase of exon per million fragments mapped (FPKM). P2B-NH, P2B-H, P3-NH and P3-H represent WallMECs cultured on P2B ECM without hormones, P2B ECM in the presence of IFPT3E2, P3 ECM without hormones and P3 ECM in the presence of IFPT3E2 respectively. The y-axis scale is unique for every milk protein gene due to the large differences in the level of expression.
Chapter Six
6.0 General discussion

6.1 The wallaby as a lactation model

The birth of an altricial wallaby places a demand on the mother for postnatal investment in the development of the neonate (Nicholas 1988, Nicholas, Simpson et al. 1997). The pouch young’s (PY) transition from a fetus-like morphology at birth to adult-like features at the onset of phase 3 (P3) happens when it is dependent only on milk (Nicholas 1988, Nicholas, Simpson et al. 1997). It is feasible therefore to suggest that the molecular signals needed for the growth and development of the neonate from parturition until it ventures out of the pouch are contained in the milk. This proposition is indeed supported by evidence showing that the growth and development of the PY from an early phase of lactation is accelerated significantly when it is fostered onto a mother producing a later stage milk (Trott, Simpson et al. 2003, Kwek, Iongh et al. 2009). It is also well known that a major part of the adaptive strategy for neonatal survival in the wallaby is the ability of the mother to progressively vary the milk composition profoundly (Nicholas 1988, Nicholas, Simpson et al. 1997) in accordance with the stage of development of the PY, in effect ensuring that milk content is ‘customised’ to the development needs of the PY.

Therefore a great advantage of the wallaby as a lactation model is that the major morphological changes that occur in the neonate can be correlated with specific changes in milk composition which occur during the transition from one phase of lactation to another (Nicholas, Simpson et al. 1997).

6.2 Key questions

The individual studies that make up this thesis collectively addressed four key questions about the wallaby lactation strategy. Firstly, how does the timing of delivery of milk bioactives in milk correlate with their function in relation to neonatal development and
mammary function? To address this question, the study examined the expression profile of MaeuCath1, a wallaby cathelicidin expressed in milk, and performed in-vitro anti-bacterial and cell proliferative activity assays to assess MaeuCath1 bioactivity. Secondly, what factors in the mammary gland regulate the timing of delivery of milk bioactives including cathelicidins? Drawing from previous studies on possible effectors of ACL (Lincoln and Renfree 1981, Nicholas 1988, Nicholas, Simpson et al. 1997, Trott, Simpson et al. 2003) and a large body of evidence suggesting that the extracellular matrix (ECM) regulates mammary epithelial cell function the ECM was investigated as a possible regulator of milk protein gene expression and delivery of bioactives in milk. The third key question addressed which elements in the cathelicidin gene are responsive to regulatory stimuli provided by the ECM. This question was addressed by transfecting WallMEC with deletion fragments of the 2245 bp sequence 5’ to the translation start site of the MaeuCath1 gene cloned in a luciferase reporter vector. The cells were cultured on ECM and gene expression assessed by performing luciferase activity assays. Lastly, what molecular pathways are regulated by the ECM leading to changes in lactation phenotype? To address this question WallMEC were cultured on ECM from P2B and P3 in either the presence or absence of lactogenic hormones and RNA isolated for transcriptome analysis.

6.2.1 MaeuCath expression and function during lactation

Eight cathelicidin genes, MaeuCath1-8, (GenBank accession numbers ABV01938.1, ABV01939.1, ABV01940.1, EF624484.1, ABV01941.1, ABV01942.1, ABV01943.1 and ACJ76797.1 respectively) have previously been reported in the wallaby (Daly, Digby et al. 2008), the first seven of which are expressed in the mammary gland. MaeuCath1 is also expressed in the lymphoid tissues of the neonate during P2A and early P2B (Daly, Digby et al. 2008) while MaeuCath8 is expressed in the spleen and gut of the neonate in the immediate postpartum period and in a number of other tissues after seven days post-partum (Carman,
Old et al. 2009, Wang, Wong et al. 2011). However upon examining the wallaby and opossum (*Monodelphis domestica*) genomic sequence in the Ensembl database, this study found that ABV01938.1 and EF624482.1 representing MaeuCath1 and MaeuCath2 respectively map to chromosome 6 of opossum with identical synteny and localise to the same gene scaffolds in the wallaby genome. Additionally they were homologous except for a retained intronic sequence and lack of exon 3 and 4 in MaeuCath2. Based on these findings this study concluded that MaeuCath1 and 2 are splice variants of the same gene. Therefore further studies may be required in order to clarify the identities of MaeuCaths genes.

The present study demonstrated that both MaeuCath1 and MaeuCath7, cathelicidins secreted in wallaby milk have antimicrobial activity in their variable C’terminal portion and the capacity to enhance mammary epithelial cell proliferation in the cathelin domain (Wanyonyi, Sharp et al. 2011). Synthetic peptides (Con73 and Con218 designed from the heterogeneous C’terminal portion of MaeuCath1 and MaeuCath7 respectively) had bactericidal activity against *Staphylococcus aureus, Pseudomonas aureginosa, Salmonella enterica* and *Enterococcus faecalis*, consistent with the need to provide antimicrobial protection to the young before it develops its own immunity (Daly, Digby et al. 2007). The ability of MaeuCath peptides to kill *S.aureus* also pointed to a possible role in the protection of the lactating mammary gland against mastitis agents (Oikonomou, Machado et al. 2012).

Although MaeuCath1 and MaeuCath7 expression had earlier been reported in wallaby mammary gland, this is the first study to demonstrate the antimicrobial activity of MaeuCaths both in the context of neonatal and mammary gland protection. However it was initially paradoxical that MaeuCath peptides could kill *E. faecalis*, a commensal of the gut until, upon examining the expression profile of MaeuCath1 it became evident that the delivery of cathelicidins through milk is temporally regulated so that they are available only when they are required. In the case of MaeuCath1, the study showed that it is regulated through alternate
splicing so that the antimicrobial domain (MaeuCath1a) is only expressed in early lactation when it is required for protection of the immune-compromised neonate (Daly, Digby et al. 2007) and at involution when the mammary gland of the mother would be vulnerable to potential mastitic infections (Williams and Patnode 1948, Sordillo, Nickerson et al. 1987). In this way the mother provides broad-spectrum antimicrobial molecules through milk without perturbing the microfloral balance of the neonate’s gut. An interesting variation of this strategy is the delivery of antimicrobials with selective bactericidal activity as was observed in the case of WAP four-disulfide core domain 2 (WFDC2) (Watt, Sharp et al. 2012). While WFDC2 had a similar expression profile to MaeuCath1a in tammar milk, its antimicrobial domain (domain II) had no bactericidal activity against *E. faecalis* but strong activity against *Staphylococcus aureus*, *Pseudomonas aureginosa* and *Salmonella enterica*.

### 6.2.1.1 MaeuCath1a

This thesis focussed on the host defence role of MaeuCath1a but this is likely to be only one of several roles it plays in mammary gland function. During involution (when MaeuCath1a is highly expressed) a large number of immune cells converge in the mammary gland akin to a wound healing environment (Findlay 1982, Schedin, O’Brien et al. 2007). Accompanying the complex milieu of pro-inflammatory activities is an elevated risk of mastitic infections (Williams and Patnode 1948, Sordillo, Nickerson et al. 1987) and a possible release of endotoxins due to bactericidal activity of immune cells. Interestingly, cathelicidins have been shown to play a role in the different levels of immune response including regulation of immune genes (Yang, Biragyn et al. 2004), chemotaxis of immune cells (Ciornei, Sigurdardottir et al. 2005) and neutralisation of endotoxins (Bhunia, Mohanram et al. 2009). Therefore it is conceivable that MaeuCath1a plays these roles in the involuting mammary gland.
Apart from the possible roles in immune response, MaeuCath1a may participate in the process of involution by directly inducing apoptosis of epithelial cells as has been demonstrated for BMAP-28, a bovine cathelicidin. BMAP-28 induces apoptosis through the initiation of the mitochondrial permeability transition pore (MPT pore) and the consequent release of cytochrome c (Risso, Braidot et al. 2002). Although the mechanism by which BMAP-28 initiates MPT is not well understood, this role is likely to link it to a number of signaling pathways related to mitochondria-mediated apoptosis. BMAP-28 may initiate cytochrome c release through interaction with the Bcl-2 family of proteins which modulate apoptosis through MPT by interacting with survival factors via their downstream signaling molecules (Lorenzo and Susin 2004, Chalah and Khosravi-Far 2008, Rong and Distelhorst 2008). For example the pro-apoptotic activity of Bad, one of the Bcl-2 family members is inhibited by survival factors including cytokines and growth factors through PI3K/Akt signaling. Conversely, death stimuli induce the expression of Bax, Noxa and PUMA (other pro-apoptotic members of the Bcl-2 family) through JNK (for Bax) and p53 signaling (Lorenzo and Susin 2004, Chalah and Khosravi-Far 2008, Rong and Distelhorst 2008, Brenner and Mak 2009, Eliseev, Malecki et al. 2009). BMAP-28 has been shown to selectively kill transformed and proliferating lymphocytes relative to normal non-proliferating cells through MPT (Zanetti 2004). If MaeuCath1a has pro-apoptotic properties similar to BMAP-28, it would be interesting to investigate it for selective killing of transformed cells and possible role in inhibition of tumour progression.

6.2.1.2 MaeuCath1b

The second splice variant, MaeuCath1b was expressed predominantly in late P2B and during P3 which represent the periods of active mammary enlargement and peak milk production (Nicholas, Simpson et al. 1997) respectively. The ability of MaeuCath1b to enhance mammary epithelial cell proliferation was consistent with the need for production of larger
volumes of milk to feed a rapidly growing neonate during P3. Interestingly, prior to the current study, tWAP, a member of the four-disulphide core domain (4-DSC) family of proteins to which WFDC2 belongs was shown to enhance mammary epithelial cell proliferation (Topcic, Auguste et al. 2009). Specifically, domain III of tWAP stimulated the proliferation of both the mouse mammary epithelial cell line (HC11) and WallMEC. Considering that tWAP is expressed mainly during P2B (Topcic, Auguste et al. 2009) and is almost undetectable at the onset of P3, it appears that while the cell-proliferative function of milk is retained, there is a change with respect to the specific peptides that carry out this function during the course of lactation.

Two questions stem from the proliferative effect of MaeuCath1b; how does MaeuCath1b stimulate MEC proliferation and is MaeuCath1b involved in cell survival signaling? In a wound healing environment the porcine cathelicidin peptide, PR-39 has been thought to signal cell proliferation by inducing the expression of syndecans (Bernfield, Kokenyesi et al. 1992), a family of heparan sulphate proteoglycans known for their regulatory roles in multiple physiological processes including cell adhesion and blood coagulation (Bernfield, Kokenyesi et al. 1992). The syndecans effect cell proliferation by sensitising cells to growth factors including the transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) (Bernfield, Kokenyesi et al. 1992, Zanetti 2004). The possibility of MaeuCath1b signaling cell proliferation through the syndecans therefore links Maeucath1b to multiple TGF-β-regulated physiological processes including angiogenesis, extracellular matrix neogenesis and immune-suppression (Ijichi, Otsuka et al. 2004). However, this proposition although feasible is somewhat controversial because in normal epithelial cells TGF-β signals apoptosis instead of proliferation (Massague 2000). Nevertheless the possible role of Maeucath1b in mammary growth during peak lactation is worth further investigation.
6.2.2 The role of the extracellular matrix (ECM) in wallaby lactation

The ability of the wallaby to initiate two parallel lactations in two adjacent mammary glands (asynchronous concurrent lactation; ACL) each with its own milk composition (Nicholas 1988) offers an incredible advantage in investigating factors that regulate milk protein gene expression. Further, the progressive changes in milk composition in the individual mammary glands is marked by phase-specific induction and silencing of specific genes, providing what has been called natural gene knockout (Brennan, Sharp et al. 2007). Therefore by performing a comparative analysis of the molecular composition of the two mammary glands involved in ACL, it is possible to identify factors that instruct the asynchronous expression of milk protein genes. In this thesis I report that the asynchronous expression of milk protein genes in the mammary glands of the wallaby is regulated by progressive changes in the extracellular matrix.

ECM extracted from a later phase of lactation changed the phenotype of WallMEC from earlier phase of lactation to resemble the later phase. For example when P2B cells were cultured on P3 ECM they expressed the late lactation protein-B (LLP-B) gene, consistent with published data showing that the ECM regulates mammary epithelial cell function (Li, Aggeler et al. 1987, Deugnier, Faraldo et al. 1999). However the ECM-directed transition of MECs from an earlier phase to a later phase appeared to proceed in a stepwise manner so that P3 ECM could not change the phenotype of P2A cells to P3. Additionally the ECM could not reverse the phenotype of WallMEC to an earlier phase phenotype. This inability of the ECM to program cells backwards or forward by more than one phase suggests that phase-specific-milk protein gene expression in wallaby is tightly regulated especially considering that in an in-vitro rat model, ECM from a late involuting mammary gland changed a culture from an alveolar structure back to a ductal structure (Schedin, Mitrenga et al. 2004). Therefore this study would have been enriched further by culturing P2A WallMEC on P2B ECM,
examining the change in phenotype and then transferring the cells onto P3 ECM, effectively confirming that change in phenotype must proceed in a stepwise manner.

The tight regulation of milk protein gene expression is consistent with the need for the mother to produce milk that matches the level of gut development at every stage of lactation. Therefore it was particularly interesting when RNA-seq data revealed that relative to P2B ECM, P3 ECM differentially up-regulated the expression of enzymes that metabolise galactose including UDP-glucose 6-dehydrogenase, glucose phosphomutase (Berg JM, Tymoczko JL et al. 2002), UDP-galactose 4-epimerase and hexokinase (Holden, Rayment et al. 2003) possibly leading to reduction in milk carbohydrate. During P3, the milk is rich in protein and lipid but low in carbohydrate and whereas the main carbohydrates in P2 milk are complex oligosaccharides, P3 milk contains mainly monosaccharides (Menzies and Nicholas 2007). Previous studies have attributed the decrease in oligosaccharides during P3 to the down-regulation of 3βGalT and 4βGalT which collectively catalyse the synthesis of oligosaccharides and the increase in the expression of UDP-galactose hydrolase which breaks down UDP-galactose, a building block of oligosaccharides (Messer and Elliott 1987, Messer and Nicholas 1991). However, prior to this thesis no association had been shown between ECM and the regulation of milk carbohydrate content during wallaby lactation.

Equally interesting was the down-regulation of ASAH1 (N-acylsphingosine amidohydrolase) by P3 ECM relative to P2B ECM as revealed by RNA-seq data. ASAH1 also known as acid ceramidase digests ceramides (Lucki, Bandyopadhyay et al. 2012) which are the major phospholipid in the bovine milk fat globule (Parodi 1997). Although the ceramide content of wallaby milk is not well documented, it is possible that during P3 the ECM regulates milk lipid content by down-regulating lipid catabolic enzymes. Consequently, this thesis is the first to report a possible association between the ECM and the elevated milk lipid content during P3.
6.2.3 ECM-regulated functional pathways in WallMEC

In this study the ECM appeared to exert a global effect on WallMEC, impacting several pathways linked to multiple cellular processes including apoptosis, proliferation, differentiation, sugar metabolism and lipid metabolism. Clearly there was a synergistic relationship between hormones and the ECM, since some pathways including the lysosome, complement and coagulation, cell adhesion molecules (CAM) and p53 signaling pathways were only responsive to ECM in the presence of hormones. Similarly, the galactose metabolism and Nod-like receptor signalling pathways had significant enrichment scores only in hormone treated samples. The implications of the impact of ECM on these signalling pathways in regard to mammary function and lactation are discussed in chapter 5, section 3.

6.2.4 What regulates ECM composition?

In this study, consistent with published evidence (Schedin, Mitrenga et al. 2004) the regulatory effect of the ECM appeared to be mediated by progressive changes in ECM composition in a lactation-phase dependent manner. ECM extracted from pregnant (P1), P2A and P2B mammary glands had predominantly intact multimeric proteins while ECM from P3 and involuting mammary glands had a significant amount of breakdown and monomeric fragments of the large ECM proteins. Gelatin zymography of the ECMS showed that the changes in ECM may be mediated both by differential expression of the ECM protein genes and increase in matrix metalloprotease (MMP) activity in late lactation and involution. Therefore, while it is clear that ECM composition controls phenotype change in WallMEC, the instructive cues that signal changes in ECM composition according to the lactation phase need to be investigated.

The concept of dynamic reciprocity between the ECM and epithelial cells suggests that the instructive cues that ultimately change ECM composition originate from the ECM (Bissell
and Aggeler 1987, Roskelley and Bissell 1995, Spencer, Xu et al. 2007, Maller, Martinson et al. 2010). Examination of the RNA-seq data in this study indeed appeared to confirm this concept. Among the differentially expressed functional gene clusters, the ECM related cluster represented at least 3% of the differentially regulated genes between P2B and P3 ECM treated WallMEC. This was true for the hormone (H) and no hormone (NH) treated cells and the differentially regulated genes included the ADAMTS’s, MMP’s, tenascin and collagens secreted by MECs, among other ECM constituent proteins. These observations are consistent with the ECM inducing the secretion of ECM-modulating proteins by cells, hence the dynamic and reciprocal relationship between ECM and epithelial cells. However, a question remains as to whether changes in ECM precede changes in the cell or the other way round.

It is possible that the overarching instructive cues that direct changes in ECM composition originate from systemic developmental signals that determine tissue specificity and organ development rather than the cell-ECM interaction. Relative to other organ systems the mammary gland is unique because the major part of its development occurs during puberty and only becomes functionally active in adulthood (Watson and Khaled 2008). Therefore it is conceivable that factors which program the development of other organs at the embryonic stage act on the mammary gland during puberty and possibly pregnancy and lactation. Understandably, the process would involve a complex set of molecular networks that are beyond the scope of this thesis. However I can speculate that development regulators including the Hedgehog (Hh) signalling network which determines organ patterning and stem cell lineage (Ingham and McMahon 2001, Watson and Khaled 2008) in embryos may play a role in specifying ECM composition. Other likely candidates include the parathyroid hormone-related protein (PTHrP), fibroblast growth factor 10 (FGF10) and the bone morphogenic protein 4 (BMP4) which collectively specify mammary bud formation in the embryo (Hens, Dann et al. 2007, Watson and Khaled 2008). PTHrP has indeed been shown to
instruct the secretion of mammary-specific mesenchyme during embryonic mammary development (Hens, Dann et al. 2007).

However it is necessary to distinguish between cues that instruct pre-pubertal development and cues that lead to post-pubertal functional differentiation (at pregnancy and during lactation). At puberty the mammary gland comes under the control of estrogen and upon maturity is subjected to cyclical changes in ovarian hormones (Hens and Wysolmerski 2005, Hinck and Silberstein 2005, Sternlicht 2006) and similarly, during gestation and lactation the mammary gland is under strict control of lactogenic hormones in a species-dependent manner (Topper and Freeman 1980, Nicholas and Tyndale-Biscoe 1985, Nicholas 1988, Nicholas, Simpson et al. 1997). Therefore it is possible that hormones play a more prominent role than other developmental signals during puberty, gestation and lactation. This suggestion is consistent with RNA-seq data in this study showing that specific ADAMTS’s, MMP’s and collagen VII were differentially expressed between the H and NH treated WallMEC.

6.3 The role of ECM in MaeuCath1 gene expression

After confirming that the ECM regulates the switch from one lactation phase to the next, it was of interest in this study to establish whether the temporal expression of MaeuCath1 was controlled by the ECM. By performing promoter activity assays on the 5’-proximal region of the MaeuCath1 ORF, the study showed that the sequence between -709 and -15 relative to the ATG start codon contained positive regulatory activity while the region between -919 and -710 contained repressor activity both of which were dependent on the ECM. Therefore, this study showed for the first time that the ECM regulates MaeuCath1 expression through regulatory elements found in 5’-untranslated region of the gene.

Previously MaeuCath1 expression in WallMEC and leucocytes has been shown to be induced by lipopolysaccharide (LPS) and lipotechoic acid (LTA) respectively (Daly, Mailer et al.
Cathelicidin expression in other species is induced by a wide variety of stimuli including, DNA damage agents, injury, a break in the permeability barrier and ER stress (Park, Elias et al. 2011) and the effect of the stimuli appears to be tissue dependent but there is no clear association between ECM and cathelicidin expression. Therefore the identification of ECM responsive sequences in the MaeuCath1 gene adds a new dimension to the regulation of cathelicidin.

6.4 Possible methodological refinement

Numerous in-vitro culture models have shown that cells express higher amounts of β-casein when cultured on floating collagen gels than when cultured on individual ECM proteins (Parry, Cullen et al. 1987), attached collagen (Enami, Yang et al. 1979, Sasaki and Enami 1996) or plastic (Emerman, Enami et al. 1977, Enami and Nandi 1978, Katiyar, Enami et al. 1978, Bisbee, Machen et al. 1979, Enami, Yang et al. 1979, Sasaki and Enami 1996). Parry et al further demonstrated the need for flotation in MEC differentiation by obtaining a high level of β-casein expression on nitrocellulose filters touching media in tissue culture chambers (Parry, Cullen et al. 1987). These experiments demonstrated that baso-lateral contact with media is necessary for basal/apical polarity and milk gene expression. In this study the ECM had no effect on the expression of the early lactation protein (tELP) probably because the expression of tELP in-vitro is dependent on baso-lateral contact with the media. Therefore, the findings of this study could have been enriched further by including a flotation component in the methodology for chapters 3 and 4.

In chapter 3, although the study reports progressive changes in ECM during lactation the differences in ECM between the P3 and involuting mammary glands was minor, judging from the silver-stained gel. This may have been due to gradual involution in the P3 mammary gland (Khalil, Digby et al. 2008) but may also have been caused by insufficient time
difference in the dates the mammary glands were extracted. This problem could be resolved by using a larger number of samples that are spread out in time. Additionally the novel ECM protein bands (P2Ax, P2Bx and INVx) were not positively identified by mass spectrometry after in-gel proteolytic digest possibly due to the resistance of ECM proteins to proteolytic digest. A chemical approach such as the Bergmann degradation (Edman 1949, Niall 1973) method might have resolved this difficulty.

It would also be possible to dissect the role of ECM in mammary function further by cloning and expressing the differentially expressed ECM protein genes and treating WallMEC from different phases of lactation with the individual ECM proteins then examining change in lactation phenotype. The recombinant ECM proteins could be added to the ECM in different combinations and changes in the response of the cells to ECM examined.

An in-vivo model of the antimicrobial activity of MaeuCath1a and the cell proliferative activity of MaeuCath1b could have significantly enriched this study. For example by generating a MaeuCath1a transgenic mouse and performing galactophore inoculation with known mastitic agents such as S.aureus, the study could have confirmed the role of MaeuCath1a in the protection of the mammary gland against mastitis. Similarly by generating a MaeuCath1b transgenic mouse and measuring changes in mammary size, survival rate of litters and performing histological studies on the mammary glands the role of MaeuCath1b in mammary growth could have been confirmed.

6.5 Conclusion

This study has shown for the first time that ACL and the delivery of domain-specific bioactive peptides including MaeuCaths in milk are regulated through temporal changes in mammary extracellular matrix during lactation.


8. Appendices
Appendix 1

Acini morphology and abundance after culturing mid lactation WallMEC’s on ECM extracted from mammary glands representing various phases of lactation.  

A: 40x magnification of acini after culturing cells for 13 days. PG, Early, Mid, Late and INV represent pregnant, early, mid and late lactation and involution respectively.  

B: Effect of ECM phase on acini morphology. On the x-axis is the phase of lactation from which mammary ECM was isolated. On the y-axis is the number of acini. Each treatment was performed in triplicate and the experiment repeated three times. Standard error bars are shown. M1-M6 represent the various acini types in order of decreasing size. Due to the significant difference in the numbers of acini subtypes the y-axis scale is specific to each acinus subtype.
Appendix 2

Milk protein gene expression after culturing mid lactation WallMEC’s on ECM extracted from mammary glands representing the various phases of lactation. On the y-axis is the gene expression level expressed as a percentage of GAPDH expression and on the x-axis is the lactation phase of the ECM. Samples were either treated with insulin and cortisol (IF) or the hormone cocktail consisting of insulin, cortisol, prolactin, tri-iodothyronine and estradiol (IFPT₃E₂). The asterisk denotes significant difference between IF and IFPT₃E₂. Samples were treated in duplicate and the experiment repeated twice. Standard error bars are shown. The y-axis scale is unique for each of the proteins due to the significant difference in their expression levels.
Appendix 3

Acini morphology and numbers after culturing early lactation WallMEC’s on ECM extracted from mammary glands representing the various phases of lactation. A: 40x magnification images of acini after culturing cells for 13 days. PG, Early, Mid, Late and INV represent phases pregnant, early, mid and late lactation and involution respectively. B: Effect of ECM phase on acini morphology. On the x-axis is the phase of lactation from which mammary ECM was isolated. On the y-axis is the number of each type of acini. Each treatment was performed in triplicate and the experiment repeated three times. Standard error bars are shown. M1-M6 represent the various acini types in order of decreasing size.
Appendix 4

*Milk protein gene expression after culturing early lactation WallMEC’s on ECM extracted from mammary glands representing the various phases of lactation.* On the y-axis is the gene expression level expressed as a percentage of GAPDH and on the x-axis is the lactation phase of the ECM. Samples were either treated with IF or (IFPT3E2). The asterisk denotes significant difference between IF and IFPT3E2. Samples were treated in duplicate and the experiment repeated twice. Standard error bars are shown. The y-axis scale is unique for each of the proteins due to the significant difference in their expression levels.
Appendix 5

Quantitative PCR of MaeuCath1 using cDNA from P2A WallMECs. Cells were cultured on ECM from different phases of lactation in the presence or absence of IFPT3E2 and RNA isolated for cDNA synthesis and qPCR. Treatments were performed in triplicates and repeated twice. Representative results with their error bars are shown.
Appendix 6

*Cytokine-cytokine receptor interaction.* Up-regulated in the P2B_NH versus P3_NH dataset (higher in P3_NH) are marked with a red star.