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The extracellular matrix regulates MaeuCath1a gene expression

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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.

1. Introduction

Cathelicidins and other host defence peptides delivered through milk constitute a significant part of neonatal innate immunity (Armogida 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 (Yang et al., 2004a; Zanetti et al., 1995) and stimulate mammary cell proliferation in a domain-specific manner (Wanyonyi 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 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 et al., 2004b) 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 foetus-like young that relies on the mother's ability to profoundly change its milk composition throughout lactation (Nicholas 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 et al., 1997; Nicholas, 1988), 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 et al., in press).

We have previously shown that the gene for MaeuCath1 is regulated through alternate splicing resulting in two variants

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MaeuCath1a and Maeucath1b (Wanyonyi et al., 2011). The expression of MaeuCath1a, the splice variant containing the antimicrobial region (Wanyonyi 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 et al., 2007) and late involution when the mammary gland environment resembles a wound-healing process (O'Brien et al., 2012; Oliver and Mitchell, 1983) 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 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 et al., 1982; Schedin 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 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).

2. Materials and methods

2.1. 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.

2.2. 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 h 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 200 M mesh and then 53 M mesh Nalgene filter. The filtrates were centrifuged at 80×*g* for 5 min and cell pellets washed twice by suspending in HBSS and centrifuging at 80×*g* for 5 min. After the final wash, cells were resuspended in freezing media (90% FCS (Invitro Technologies)/10%DMSO (Sigma, D8418), at a density 2×10^7 cells/ml and frozen in liquid nitrogen.

Table 1

Name	Primer sequence (5′–3′)	Restriction enzyme site
Primers for amplifying MaeuCath1 cDNA		
MaeuCath1 Fwd	ATGCAGGTACTCCTATTGGTG	
MaeuCath1 Rev	CCCTGGCAGTGGGATAGGAAT	
Primers for generating promoter deletions		
−2245 Forward	gcaatcCTCGAGTGTTCTGGTGCTAAAAGTCTGG	Xho I
−1885 Forward	gcaatcCTCGAGCAGGCAAGAGGAGTAAAATG	Xho I
−1577 Forward	gcaatcCTCGAGTCACCTCAGTACAATGCCTG	Xho I
−1260 Forward	gcaatcCTCGAGTGTTGGGAGTTCTTCTTTAC	Xho I
−919 Forward	gcaatcCTCGAGTACATCTCAATTGGTGTCAAG	Xho I
−709 Forward	gcaatcCTCGAGAATACCTCAGTTTCACGCCCA	Xho I
−484 Forward	gcaatcCTCGAGCTGAGCATTTGGGAAGGATT	Xho I
Common Reverse	ggatctAAGCTTATGCTCTGGGCAGAAAGAA	Hind III
Primers for cloning MaeuCath1 promoter fragments into Rβ-casein promoter construct		
−1260 Forward	gcaatcCTCGAGTGTTGGGAGTTCTTCTTTAC	Xho I
−919 Reverse	ggatctAAGCTTGCTATCTCTCATTCACTGCTG	Hind III
−709 Forward	gcaatcCTCGAGAATACCTCAGTTTCACGCCCA	Xho I
−709 Reverse	ggatctAAGCTTGCCACAGGTTACTAATTAGTC	Hind III
Primers for gel shift assay		
GS1F	CATATATGACATTCCTCTGCCACCTGTTTCAGCAGTGAATGAGAGATAGC	
GS1R	GCTATCTCTCATTCACTGCTGAAACAGGTGGCAGAGGAATGTCATATATG	
GS2F	ATTTACTACCCAAGGAATAGTTCCTTCCTTCTCCAGTTGACATATATGAC	
GS2R	GTCATATATGTCAACTGGAGAAGGAAGGAACTATTCCTTGGGTAGTAAAT	
GS3F	GTCAAATTTGGACTAGGACTTTTCTCCTTGCTTTCTATCCATTTACTACC	
GS3R	GGTAGTAAATGGATAGAAAGCAAGGAGAAAAGTCCTAGTCCAAATTTGAC	
GS4F	CATCCTTTATGTTGGGGTAGGAGTCCAATATAATCCATTTGTCAAATTTG	
GS4R	CAAATTTGACAAATGGATTATATTGGACTCCTACCCCAACATAAAGGATG	
GS5F	TACATCTCAATTGGTGTCAAGACAAGTGCATATAAATTTGCATCCTTTAT	
GS5R	ATAAAGGATGCAAATTTATATGCACTTGTCTTGACACCAATTGAGATGTA	
Primers for excluding repressor fragment from MaeuCath1 promoter (the region between −709 and −919)		
−919 rep Rev	ggatctCTCGAGACAATGGCTTGTCATTATGAG	Xho I
−1260 rep Fwd	gcaatcGGTACCTGTTGGGAGTTCTTCTTTAC	Kpn 1
−2245 rep Fwd	gcaatcGGTACCTGTTCTGGTGCTAAAAGTCTGG	Kpn 1

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.