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Mammary cell-activating factor regulates the hormone-independent transcription of the *early lactation protein* (*ELP*) gene in a marsupial

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ABSTRACT

The regulation of the tammar wallaby (*Macropus eugenii*) *early lactation protein* (*ELP*) gene is complex. *ELP* is responsive to the lactogenic hormones; insulin (I), hydrocortisone (HC) and prolactin (PRL) in mammary gland explants but could not be induced with lactogenic hormones in tammar primary mammary gland cells, nor in KIM-2 conditionally immortalised murine mammary epithelial cells. Similarly, *ELP* promoter constructs transiently-transfected into human embryonic kidney (HEK293T) cells constitutively expressing the prolactin receptor (PRLR) and Signal Transducer and Activator of Transcription (STAT)5A were unresponsive to prolactin, unlike the rat and mouse *β-casein* (*CSN2*) promoter constructs. Identification of the minimal promoter required for the hormone-independent transcription of tammar *ELP* in HEK293Ts and comparative analysis of the proximal promoters of marsupial *ELP* and the orthologous eutherian colostrum trypsin inhibitor (*CTI*) gene suggests that mammary cell-activating factor (MAF), an E26 transformation-specific (ETS) factor, may bind to an AGGAAG motif and activate tammar *ELP*.

1. Introduction

The tammar wallaby (*Macropus eugenii*) has a short pregnancy of ~26.5 days and gives birth to an altricial young. A long and complex ~300-day lactation period follows during which the composition of milk proteins, carbohydrates and lipids change dynamically so as to provide appropriate nutrition for the growth and development of the pouch young (Green, 1984; Nicholas et al., 1997; Sharp et al., 2009). In contrast, apart from the secretion of colostrum for the first 24–36 h postpartum (pp), the changes in mature eutherian milk are generally less dramatic (Jenness, 1974; McSweeney and Fox, 2013).

In most mammals, the major casein and whey protein genes including: *α-* and *β-casein* (*CSN1*, *CSN2*) and *α-lactalbumin* and *β-lactoglobulin* (*LALBA* and *LGB*) respectively, are induced at parturition and expressed throughout lactation (McSweeney and Fox, 2013; Nicholas et al., 1997). Unlike in eutherians, several key milk protein genes are temporally-expressed in marsupials. For the tammar, these include: *early lactation protein (ELP)*, *whey acidic protein* (*WAP*) and *late lactation proteins A and B* (*LLPA* and *LLPB*). *Early lactation protein* is expressed at low levels from day 10 of pregnancy, upregulated at parturition and expressed during the first third of lactation until ~125 days postpartum (pp) (Phase 2A, early lactation) (Nicholas et al., 1997; Simpson et al., 1998). The cessation of *ELP* expression and secretion coincides with the maturation of the acquired immune system and the gut-associated lymphoid tissue of the young (Joss et al., 2009). The presence of a single bovine pancreatic trypsin inhibitor (BPTI)/Kunitz domain suggests that ELP may prevent the degradation of immunoglobulins that are transferred from mother to young during this period (Piotte and Grigor, 1996; Simpson et al., 1998). Unlike *ELP*, *WAP* is expressed during Phase 2B (mid-lactation) (Simpson et al., 2000) and *LLPA* and *LLPB* are expressed during late Phase 2B-Phase 3 (late lactation) and Phase 3, respectively (Nicholas et al., 1997; Trott, 1999). The stage-specific expression of these genes suggests that they are controlled by sophisticated regulatory mechanisms.

The endocrine regulation of tammar *ELP* has been investigated using a mammary gland explant culture system (Pharo, 2014; Simpson, 1998). *ELP* is maximally responsive to the lactogenic hormones insulin (I), hydrocortisone (HC) and prolactin (PRL) *in vitro* (Pharo, 2014; Simpson, 1998). This is consistent with the

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Abbreviations

AP	activating protein
BPTI	bovine pancreatic trypsin inhibitor
CAMP	cathelicidin antimicrobial peptide
CAT	chloramphenicol acetyltransferase
C/EBP	CCAAT/enhancer binding protein
CREB	cAMP response element binding protein
CMV	cytomegalovirus
CoRE	composite response element
CSN1	*α-casein*
CSN2	*β-casein*
CTF	CCAAT box transcription factor
CTI	colostrum trypsin inhibitor
D	dexamethasone
d2EGFP	destabilised enhanced green fluorescent protein
DRE	distal regulatory element
ECM	extracellular matrix
ELF	E47-like factor
ELP	early lactation protein
ERE	estrogen response element
EST	expressed sequence tag
ERRα/β	estrogen-related receptor alpha/beta
ETS	E26 transformation-specific
EVI1	Ectopic Virus Integration site 1
GR	glucocorticoid receptor
½GRE	glucocorticoid response element
H3Ac	histone H3 acetylation
H3K4me2	histone H3 lysine 4 di-methylation
HC	hydrocortisone
I	insulin
IFNɣ	interferon gamma
IRE	insulin response element
IRF	Interferon regulatory factor
JAK2	Janus kinase 2
L	lactation
LALBA	*α-lactalbumin*
LGB	*β-lactoglobulin*
LLP	*late lactation protein*
MaeuCath	tammar wallaby cathelicidin
MAF	mammary cell-activating factor
MEC	mammary epithelial cell
MGF	mammary gland factor
MZF	Myeloid zinc finger
ncRNA	non-coding RNA
NFAT	Nuclear activator of T-cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFY	nuclear factor Y
OCT	octamer
P	pregnancy
PMF	pregnancy-specific mammary nuclear factor
PMGC	primary mammary gland cells
pp	postpartum
PRL	prolactin
PRLR	prolactin receptor
SP	specificity protein
STAT	Signal Transducer and Activator of Transcription
TFBS	transcription factor binding site
TSS	transcription start site
UTR	untranslated region
WAP	whey acidic protein
WPPCS	whey protein promoter core sequence
YY1	Yin-Yang 1

in vivo induction of *ELP* at parturition when hormones such as prolactin are elevated (Hinds, 1988; reviewed in Shaw and Renfree, 2006). Likewise, the major tammar casein and whey protein genes, i.e. *CSN1*, *CSN2*, *LALBA* and *LGB* are up-regulated at parturition (Nicholas et al., 1997; Sharp et al., 2009). Notably, *ELP* can only be induced in explants *in vitro* if the gene is already expressed *in vivo*, i.e. in tissues from Phase 1 (pregnancy) and Phase 2A (early lactation) (Pharo, 2014; Simpson, 1998). However, *ELP* is unresponsive to lactogenic hormones in mid-and late lactation explants (Pharo, 2014). Interestingly, *ELP* expression can be maintained in Phase 1 explants treated with either insulin or insulin and hydrocortisone (Pharo, 2014), unlike many milk protein genes which require lactogenic hormones, or prolactin at least, to initiate gene transcription (Brisken and Ataca, 2015; Qian and Zhao, 2014; Rosen et al., 1999).

There are many factors that influence the expression of milk protein genes. These include: *cis*- and *trans*-acting transcription factors, distal and/or intragenic enhancers, non-coding RNAs (ncRNA), post-transcriptional regulation, extracellular matrix (ECM) factors, cell-cell interactions and hormones (e.g. insulin, glucocorticoids and prolactin) [see reviews (Qian and Zhao, 2014; Rijnkels et al., 2010; Rosen et al., 1999)]. Global and/or local chromatin organisation also plays an important role in the stage and tissue-specific expression of mammary gland genes (Rijnkels et al., 2010, 2013).

Although the mechanisms that regulate many milk protein genes in eutherians have been investigated (Qian and Zhao, 2014; Rijnkels et al., 2010; Rosen et al., 1999), those that control the temporal, mammary gland-specific expression of *ELP* are unknown. We used primer extension analysis, promoter reporter assays and comparative genomics analyses of *ELP* and the orthologous eutherian *colostrum trypsin inhibitor* (*CTI*) gene (Pharo et al., 2012) to identify conserved transcription factor binding sites that may regulate the temporal expression of tammar *ELP*.

2. Materials and methods

2.1. Animals

Tammar wallabies (*Macropus eugenii*) were kept in grass paddocks with *ad libitum* access to food, water and shelter in accordance with the National Health and Medical Research Council guidelines (NHMRC, 2013). The collection of tammar mammary gland tissues was approved by The University of Melbourne Animal Experimentation Ethics Committee.

2.2. Primer extension analysis of tammar ELP

The transcription start site (TSS) of the tammar *ELP* gene was identified using the Primer Extension System AMV Reverse Transcriptase kit (Promega Corporation) according to the manufacturer's instructions. The *ELP* TSS was determined at three different time points during pregnancy (P) and lactation (L): 1. day 25P (Phase 1, pregnancy), 2. day 80L (Phase 2A, early lactation) and 3. day 260L (Phase 3, late lactation, negative control). A 30 bp [γ-^{32}P]-ATP end-labelled *ELP* reverse primer 5′-GCGAAGTAGAGGGCA-