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Expression of the mammary gland-specific tammar wallaby *early lactation protein* gene is maintained *in vitro* in the absence of prolactin

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ABSTRACT

Marsupial *ELP* (*early lactation protein*) and its eutherian orthologue, *CTI* (*colostrum trypsin inhibitor*) are expressed in the mammary gland only for the first 100 days postpartum (Phase 2A) in the tammar wallaby and during the bovine and canine colostrogenesis period 24–36 h postpartum respectively. The factors which regulate temporal *ELP* and *CTI* expression are unknown. A tammar mammary gland explant culture model was used to investigate *ELP* gene regulation during pregnancy and early- and mid-lactation (Phase 1, 2A and 2B respectively). Tammar *ELP* expression could only be manipulated in explants *in vitro* if the gene was already expressed *in vivo*. *ELP* expression was maximal in Phase 1 explants treated with lactogenic hormones (insulin, hydrocortisone and prolactin), but unlike *LGB* (*β-lactoglobulin*), *ELP* expression was maintained in insulin or insulin and hydrocortisone over a 12-day culture period. In contrast, *ELP* was down-regulated when cultured without hormones. *ELP* could not be induced in explants cultured from mid-lactation which suggested that transcriptional repressors may prevent *ELP* expression during this period.

1. Introduction

The expression of the marsupial *early lactation protein* (*ELP*) gene and the orthologous eutherian *colostrum trypsin inhibitor* (*CTI*) gene (Pharo et al., 2012) is mammary gland-specific and restricted to early lactation in marsupials (De Leo et al., 2006; Demmer et al., 1998; Nicholas et al., 1997; Pharo et al., 2012; Piotte and Grigor, 1996; Simpson, 1998), and to the brief colostrogenesis period in the cow (Laskowski and Laskowski, 1951; Veselsky et al., 1978) and dog (Pharo et al., 2012). This period coincides with the absence of an acquired immune system in the young. However, not all eutherians have a functional *CTI* gene. While the Carnivora (dog, cat, giant panda) and Cetartiodactyla (cow, pig, common bottle-nosed dolphin) have a protein-coding *CTI* gene, it has become a pseudogene in humans and other primates, rodents, the horse, elephant, and sloth (Pharo et al., 2012).

The function(s) of ELP and CTI are unknown, but these small ~10–20 kDa N-glycosylated whey proteins (Joss et al., 2009; Laskowski and Laskowski, 1951; Piotte and Grigor, 1996; Simpson et al., 1998) have a single Kunitz/BPTI (bovine pancreatic trypsin inhibitor) domain indicative of serine protease inhibitor activity (Laskowski and Kato, 1980; Rawlings, 2010). Laskowski and Laskowski (1951) hypothesized that bovine CTI prevents the proteolysis of immunoglobulins transferred from mother to calf in colostrum, whilst Piotte and Grigor (1996) suggested that brushtail possum ELP protects the young against pathogens. Alternatively, the oligosaccharides attached to ELP and CTI may act as soluble receptor analogues for bacterial and viral pathogens, preventing their passage into the gastrointestinal tract of the young (Pharo et al., 2012).

The mechanisms that regulate the temporal expression of *ELP* and *CTI* are yet to be determined, but the induction of tammar wallaby (*Macropus eugenii*) *ELP* at parturition (Pharo et al., 2012) suggests that this gene, like most milk protein genes, responds to a lactogenic complex of hormones (insulin, hydrocortisone and prolactin) at parturition (Topper and Freeman, 1980). In contrast to eutherians (Neville et al., 2002; Pang and Hartmann, 2007), the

Abbreviations: CAL, concurrent asynchronous lactation; CSN1, α-casein; CSN2, β-casein; CTI, colostrum trypsin inhibitor; E_2, 17β-oestradiol; EGF, epidermal growth factor; ELP, early lactation protein; HC, hydrocortisone; GR, glucocorticoid receptor; I, insulin; *INS*, insulin gene; IGF, insulin-like growth factor; IR, insulin receptor; LALBA, α-lactabumin; LGB, β-lactoglobulin; LLP, late lactation protein; M199, Medium 199; MAPK, mitogen-activated protein kinase; MEC, mammary epithelial cell; NS, not significant; Ph., Phase; PI3K, (phosphatidylinositol-3-kinase)/AKT; pp, postpartum; preg, pregnancy; PRL, prolactin; PRLR, prolactin receptor; STAT, signal transducer and activator of transcription; T_3, triiodothyronine; T_4, thyroxine, tetraiodothyronine.

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withdrawal of progesterone is unnecessary for tammar parturition and lactation to proceed (Hinds and Tyndale-Biscoe, 1982a; Renfree, 1994; Tyndale-Biscoe and Renfree, 1987; Tyndale-Biscoe et al., 1984; Ward and Renfree, 1984).

The tammar and eutherian milk protein genes including α-*casein* (*CSN1*), β-*casein* (*CSN2*), α-*lactalbumin* (*LALBA*) and β-*lactoglobulin* (*LGB*, a pseudogene in primates and rodents) are induced at parturition and expressed throughout lactation (Houdebine, 1999; Nicholas et al., 1997; Rudolph et al., 2003). However, tammar *ELP* (Pharo et al., 2012; Simpson et al., 1998), *whey acidic protein* (*WAP*) (Simpson et al., 2000), and *late lactation protein A* (*LLPA*) and *LLPB* (Trott et al., 2002) are asynchronously expressed at specific times during lactation, correlated with the various developmental milestones in the pouch young (Fig. 1), (Findlay and Renfree, 1984; Trott et al., 2003; Tyndale-Biscoe and Renfree, 1987).

The tammar has a short pregnancy of 26.5 days (Phase 1) and gives birth to an altricial ~440 mg young (Menzies et al., 2012; Renfree et al., 2011, 1989; Tyndale-Biscoe and Renfree, 1987). During the next ~300–350 days of its comparatively long lactation, the mother progressively alters milk production and composition to provide appropriate nutrition for the completion of organogenesis and the neurological and physiological development and growth of the young (Fig. 1) (Deane et al., 1990; Green, 1984; Green et al., 1980; Janssens et al., 1997; Tyndale-Biscoe and Janssens, 1988). Three phases of tammar lactation have been defined, Phase 2A (0–100 days postpartum (pp)), Phase 2B (100–200 days pp) and Phase 3 (200–350 days pp) (Brennan et al., 2007; Nicholas et al., 1997). After birth, the immunologically naive neonate (Edwards et al., 2012; Tyndale-Biscoe and Renfree, 1987) climbs into its mother's pouch where it remains permanently attached to one of the four teats for the next 100 days (Phase 2A, *ELP* gene expressed) (Pharo et al., 2012; Simpson, 1998). Lactation continues in the sucked gland, but ceases in the others (Findlay and Renfree, 1984; Stewart, 1984). From days 100–200 pp (Phase 2B, *WAP* expressed) (Simpson et al., 2000), the young is intermittently attached to the teat, organ development is completed and the young develops fur and homeothermy (Menzies et al., 2012; Tyndale-Biscoe and Renfree, 1987). Around 200 days pp, the young makes its initial exit from the pouch, analogous to birth in precocious eutherians (Menzies et al., 2012) and begins to eat herbage (Phase 3, *LLPA* and *LLPB* expressed) (Collet et al., 1989; Trott et al., 2002). Weaning occurs ~300–350 days pp and the mammary gland is remodelled in preparation for the next lactation cycle (Findlay, 1982a).

The endocrine regulation of some tammar milk protein genes has been characterised using an *in vitro* mammary gland explant culture model system (Brennan et al., 2008b; Simpson, 1998; Simpson et al., 2000; Trott, 1999), similar to those used to characterise milk protein gene regulation in eutherian species, such as the cow, goat, rabbit, guinea pig, rat and mouse (Burditt et al., 1981; Kulski et al., 1983; Puissant and Houdebine, 1991; Skarda et al., 1982). These 3D models are generally superior to primary and immortalised cell lines as the tissue-specific architecture, e.g. basoapical polarity of mammary epithelial cells (MECs) (Brennan et al., 2008b) and signalling between MECs and the extracellular matrix are preserved *in vitro* (Casey et al., 2000; Emerman et al., 1977; Lelievre et al., 1996).

Maximal *in vitro* expression of tammar *CSN1*, *CSN2* (Collet et al., 1992; Nicholas et al., 1995), *LGB* (Nicholas and Tyndale-Biscoe, 1985), *ELP* (Simpson, 1998), *WAP* (Simpson et al., 2000) and *LLPA* and *LLPB* (Trott et al., 2002; Trott, 1999) requires the synergistic

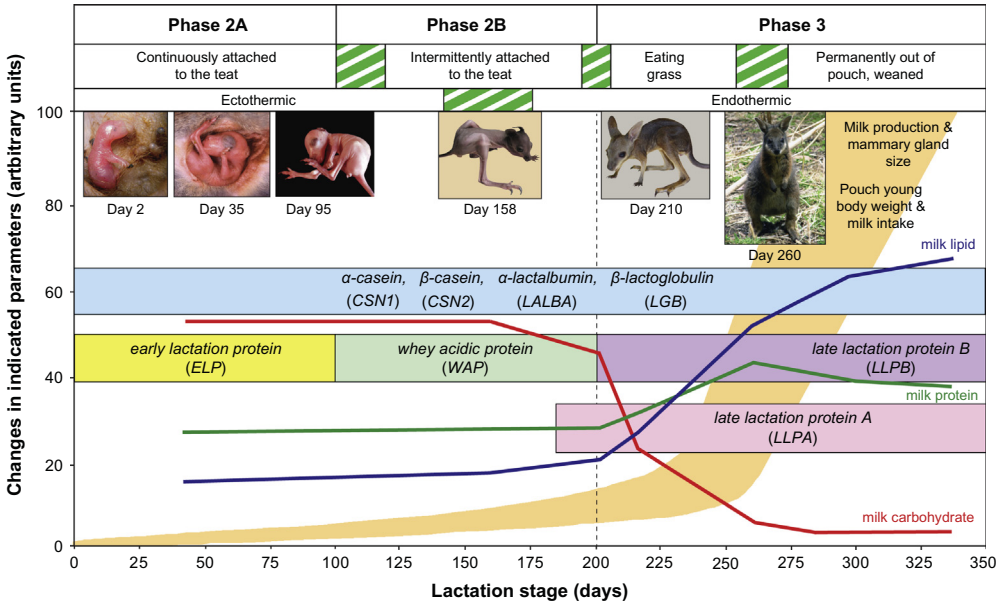

Fig. 1. Expression of the major milk protein genes and development of the pouch young during the reproductive cycle of the tammar wallaby. The tammar has a short pregnancy of ~26.5 days and gives birth to an altricial young. For the next 300–350 days the mother alters milk composition (protein, lipid and carbohydrate) and volume to provide for the growth and development of the pouch young (Findlay, 1982b; Green et al., 1983; Renfree et al., 1989; Shaw and Renfree, 2006; Tyndale-Biscoe and Renfree, 1987). Tammar lactation comprises three phases: Phase 2A, 2B and 3. Each phase is correlated with altered sucking patterns, changes in pouch young physiology and development (green and white striped boxes) (Tyndale-Biscoe and Janssens, 1988; Tyndale-Biscoe and Renfree, 1987) and changes in milk protein gene expression (Nicholas et al., 1997). The young spends the first 200 days in the pouch, continuously (0–100 days pp) and then intermittently attached to the teat (100–200 days pp). Phase 3 commences ~200 days pp and the fully developed young emerges from the pouch and starts to ingest herbage to supplement its milk intake. During this period, there is a dramatic increase in the body weight and milk intake of the young and in maternal mammary gland size and milk production (orange curve) (Findlay, 1982b). Permanent pouch exit occurs ~250 days pp and weaning ~300–350 days pp. As for eutherians, α-casein (*CSN1*), β-casein (*CSN2*), α-lactalbumin (*LALBA*) and β-lactoglobulin (*LGB2*) are induced at parturition and expressed throughout lactation. However, *ELP* is expressed during Phase 2A only, *WAP* during Phase 2B, and Late lactation proteins A and B (*LLPA*, *LLPB*) during late-Phase 2B-Phase 3 and Phase 3 respectively (Collet et al., 1989; Menzies and Nicholas, 2007; Nicholas et al., 1987, 1994, 1995, 1997; Nicholas, 1988a,b; Pharo et al., 2012; Simpson et al., 1998, 2000; Trott et al., 2002; Trott, 1999). Adapted from Green and Merchant (1988) and Green et al. (1983). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)