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REVIEW

Post-natal imprinting: evidence from marsupials

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Genomic imprinting has been identified in therian (eutherian and marsupial) mammals but not in prototherian (monotreme) mammals. Imprinting has an important role in optimising pre-natal nutrition and growth, and most imprinted genes are expressed and imprinted in the placenta and developing fetus. In marsupials, however, the placental attachment is short-lived, and most growth and development occurs post-natally, supported by a changing milk composition tailor-made for each stage of development. Therefore there is a much greater demand on marsupial females during post-natal lactation than during pre-natal placentation, so there may be greater selection for genomic imprinting in the mammary gland than in the short-lived placenta. Recent studies in the tammar wallaby confirm the presence of genomic imprinting in nutrient-regulatory genes in the adult mammary gland. This suggests that imprinting may influence infant post-natal growth via the mammary gland as it does pre-natally via the placenta. Similarly, an increasing number of imprinted genes have been implicated in regulating feeding and nurturing behaviour in both the adult and the developing neonate/offspring in mice. Together these studies provide evidence that genomic imprinting is critical for regulating growth and subsequently the survival of offspring not only pre-natally but also post-natally.
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INTRODUCTION

Genomic imprinting has been identified in all eutherian and marsupial species examined. Currently, about 150 autosomal and X-linked imprinted genes have been identified in eutherians (Williamson *et al.*, 2013). Of these, approximately 29 have been investigated in marsupials, excluding X-inactivation genes *Xist* (Davidow *et al.*, 2007) and *RSX* (Grant *et al.*, 2012). So far 8 have been confirmed to be imprinted, 13 are thought not to be imprinted and 8 genes appear to be absent from the marsupial genome (Pask, 2012; Renfree *et al.*, 2013; Table 1). It should be noted that non-conventional, outbred animals models have significant limitations for imprinting analyses. Thus, some studies use non-quantitative methods to analyse imprinted gene expression, and many examine whole-organ homogenates in which different cell types may have imprinted and non-imprinted expression patterns that can lead to the erroneous conclusion that imprinted expression does not occur.

Although expression studies in monotremes are limited to two genes so far, insulin-like growth factor 2 and one of its receptors (*IGF2* and *IGF2R*, respectively), additional bioinformatic analysis suggests that imprinting does not exist in the monotreme mammals (Pask *et al.*, 2009; Renfree *et al.*, 2009; Suzuki *et al.*, 2011a). Thus, imprinting is presumed to have first evolved concurrently with viviparity in mammals (Renfree *et al.*, 2013), after the divergence of the monotreme lineage from the therian mammals but before the divergence of eutherian and marsupial mammals around 160 million years ago (Luo *et al.*, 2011). However, the only tissues tested for imprinting in monotremes have been the adult brain, intestine, liver, kidney and spleen (Killian *et al.*, 2000, 2001; Edwards *et al.*, 2007). In the mouse, for example, *IGF2* expression in the adult is almost entirely restricted to the choroid plexus where it is biallelically expressed. Similarly, tammar *IGF2* imprinting may be developmental age specific (Suzuki *et al.*, 2005; Smits *et al.*, 2008; Stringer *et al.*, 2012b), as it is in humans (Issa *et al.*, 1996; Li *et al.*, 1998), so it remains possible that there are imprinted genes in monotremes.

Imprinting negates the advantages of diploidy by causing mono-allelic gene expression, consequently increasing the chance of exposing deleterious mutations. Many hypotheses have been developed attempting to explain how and why imprinting evolved. However, no single theory can explain unequivocally the existence of imprinting at all loci, and imprinting may have evolved at different loci under different evolutionary pressures.

Marsupials such as the Australian tammar wallaby (*Macropus eugenii*) and the South American opossum (*Monodelphis domestica*) have proven to be useful comparative models in which to investigate the evolution and maintenance of imprinted genes (Pask, 2012; Graves and Renfree, 2013; Renfree *et al.*, 2013). Analysis of the marsupial paternally expressed gene 10 (*PEG10*), the ubiquitin-protein ligase E3A (*UBE3A*)/small nuclear ribosnucleoprotein polypeptide N (*SNRPN*) region and growth factor receptor binding protein 10 (*GRB10*) supports the host-defence hypothesis for the acquisition of imprinting (for more information, see Renfree *et al.*, 2009, 2013). The host-defence hypothesis proposes that therian

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Table 1 Genes imprinted in eutherians that have been examined in marsupials

Gene	*Eutherian study*	*Marsupial study*
Genes imprinted in marsupials		
HTR2A	De Luca *et al.* (2007). *Psychiatry Res* **151**(3): 243–248.	Das *et al.* (2012). *BMC Genomics* **13**: 394.
H19	Zhang *et al.* (1992). *Nat Genet* **1**(1): 40–44.	Smits *et al.* (2008). *Nat Genet* **40**(8): 971–976.
IGF2	DeChiara *et al.* 1991. *Cell* **64**(4): 849–859.	O'Neill *et al.* (2000). *Dev Genes Evol* **210**: 18–20.
IGF2R	Barlow *et al.* (1991). *Nature* **349**(6304): 84–87.	Killian *et al.* (2000). *Mol Cell* **5**(4): 707–716.
INS	Moore *et al.* (1991). *Trends Genet* **7**: 45–49.	Ager *et al.* (2007). *Dev Biol* **309**: 317–328.
L3MBTL	Li *et al.* (2004). *PNAS* **101**(19): 7341–7346	Das *et al.* (2012). *BMC Genomics* **13**: 394.
MEST/PEG1	Kaneko-Ishino *et al.* (1995). *Nat Genet* **11**(1): 52–59.	Suzuki *et al.* (2005). *Mech Dev* **122**: 213–222.
PEG10	Ono *et al.* (2001). *Genomics* **73**(2): 232–237.	Suzuki *et al.* (2007). *PLoS Genet* **3**: e55.
Genes thought not to be imprinted in marsupials		
ASB4	Mizuno *et al.* (2002). *Biochem Biophys Res Commun* **290**(5): 1499–1505.	Suzuki *et al.* (2007). *PLoS Genet* **3**: e55.
CDKN1C	Hatada *et al.* (1995). *Nat Genet* **11**(2): 204–206	Suzuki *et al.* (2005). *Mech Dev* **122**: 213–222.
COPG2	Blagitko *et al.* (1999). *Hum Mol Genet* **8**(13): 2387–2396.	Das *et al.* (2012). *BMC Genomics* **13**: 394.
DIO3	Tsai *et al.* (2002). *Curr Biol* **12**(14): 1221–1226.	Edwards *et al.* (2008). *PLoS Biol* **6**(6): e135.
DLK1	Wylie *et al.* (2000). *Genome Res* **10**(11): 1711–1718.	Weidman *et al.* (2006). *Mamm Genome* **17**(2): 157–167.
GRB10	Miyoshi *et al.* (1998). *PNAS* **95**(3): 1102–1107.	Sringer *et al.* (2012). *Mol Biol Evol* **29**(12): 3711–3719.
IMPACT	Hagiwara *et al.* (1997). *PNAS* **94**(17): 9249–9254	Das *et al.* (2012). *BMC Genomics* **13**: 394.
PHLDA2 (*IPL*)	Qian *et al.* (1997). *Hum Mol Genet* **6**(12): 2021–2029.	Suzuki *et al.* (2011). *BMC Evol Biol* **11**: 244.
PLAGL1	Valleley *et al.* (2007). *Hum Mol Genet* **16**(8): 972–981.	Das *et al.* (2012). *BMC Genomics* **13**: 394.
PPP1R9A	Nakabayashi *et al.* (2004). *J Med Genet* **41**(8): 601–608.	Suzuki *et al.* (2007). *PLoS Genet* **3**: e55.
SGCE	Müller *et al.* (2002). *Am J Hum Genet* **71**(6): 1303–1311.	Suzuki *et al.* (2007). *PLoS Genet* **3**: e55.
SNRPN	Leff (1992). *Nat Genet* **2**: 259–264.	Rapkins *et al.* (2006). *PLoS Genet* **2**(10): e182.
UBE3A	Herzing *et al.* (2002). *Hum Mol Genet* **11**(15): 1707–1718.	Rapkins *et al.* (2006). *PLoS Genet* **2**(10): e182.
Genes with no marsupial othologues		
Air	Wutz *et al.* (1997). *Nature* **389**(6652): 745–749.	Weidman (2006). *Epigenetics* **1**(1): 49.
	Lyle *et al.* (2000). *Nat Genet* **25**(1): 19–21.	
MAGEL2	Boccaccio *et al.* (1999). *Hum Mol Genet* **8**: 2497–2505	Rapkins *et al.* (2006). *PLoS Genet* **2**(10): e182.
MEG3	Miyoshi *et al.* (2000). *Genes Cells* **5**(3): 211–220.	Weidman *et al.* (2006). *Mamm Genome* **17**(2): 157–167.
MKRN3	Jong *et al.* (1999). *Hum Mol Genet* **8**: 795–803.	Rapkins *et al.* (2006). *PLoS Genet* **2**(10): e182.
NDN	MacDonald *et al.* (1997). *Hum Mol Genet* **6**: 1873–1878	Rapkins *et al.* (2006). *PLoS Genet* **2**(10): e182.
NNAT	Kagitani *et al.* (1997). *Nucleic Acids Res* **25**(17): 3428–3432.	Evans *et al.* (2005). *Mol Biol Evol* **22**(8): 1740–1748.
PEG3	Kuroiwa *et al.* (1996). *Nat Genet* **12**(2): 186–190.	Suzuki *et al.* (2011). *GBE* **3**: 1276–1283.
RTL1 (*PEG11*)	Seitz *et al.* (2003). *Nat Genet* **34**(3): 261–262.	Davis *et al.* (2005). *Curr Biol* **15**(8): 743–749

genomic imprinting mediated by differentially methylated regions (DMRs) could originate from the cellular mechanisms such as DNA methylation that evolved to repress exogenous DNA sequences inserted by retrotransposons (Chai *et al.*, 2001; Rapkins *et al.*, 2006; Suzuki *et al.*, 2007; Stringer *et al.*, 2012a). Such genome invasions occur continuously and stochastically and attract DNA methylation. If such insertions create or entrap a gene that confers a fitness advantage in the imprinted state, it can become fixed. Thus, the host-defence hypothesis attempts to explain how imprinting is established at a locus and is distinct from the kinship and co-adaptation hypothesis, which discuss the selective pressures that are required for maintaining imprinted gene expression after its acquisition.

The vast majority of studies of imprinting in eutherians have focused on embryonic development and placental function, as differentiation occurs during gestation. In eutherians, the birth weight is usually a high proportion of the maternal body weight—for example, offspring of humans, elephants and whales are approximately 3–5% of the mother's weight. In contrast, the altricial young of the tammar is $<0.1\%$ (Table 2). Therefore there has been a greater selection pressure on a eutherian mother to provide nutrients during pregnancy than on a marsupial mother. In marsupials, the altricial neonate is tiny and is essentially an exteriorised fetus and the majority of growth occurs post-natally totally dependent on the supply of maternal milk through a long, complex and physiologically sophisticated period of lactation (Tyndale-Biscoe and Renfree, 1987; Renfree, 2010) (Figure 1). This review discusses recent studies investigating the acquisition of imprinted genes and genes with post-natal functions and relates this new information to the current theories on the evolution of imprinting.

Table 2 Gestation length and comparison of infant birth weight to maternal weight

	Elephant	*Macaque*	*Pig*	*Rat*	*Tammar*
Maternal weight, kg	2748.0	6.8	75.0	0.17	5
Approximate birth weight	120.0 kg	0.54 kg	1.45 kg	5 g	0.4 g
Average litter size	1	1	4	7	1
Duration of gestation (days)	645.4	171.2	131.3	22.3	26.5
Percentage of maternal weight	4–5	7–8	7–8	20–21	0.01

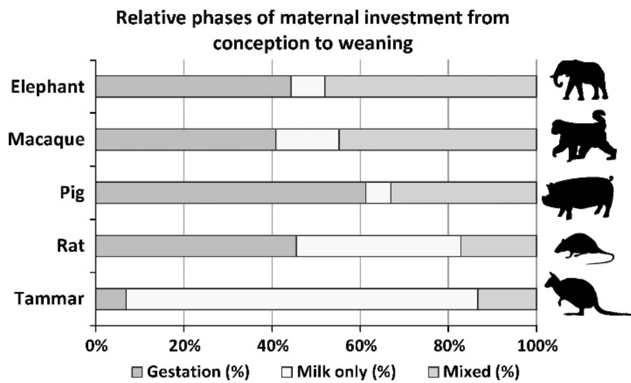

Figure 1 Relative phases of maternal investment from conception to weaning. The percentage of time spent during gestation, lactation (milk-only period) and mixed feeding (milk and solids) for elephants, macaques, pigs, rats (Langer, 2008) and tammar wallabies. There are four classes by which eutherians are grouped based on the characteristics of the young or litter at birth. Elephants represent group 4 and are described as precocial (open eyes and haired) and nidifugous (leaves the nest shortly after hatching or birth). Macaques represent group 3 and are described as precocial and transported (young are supported or carried). Pigs represent group 2 and are described as precocial and nidicolous (dependent on parent for feeding, care and protection). Rats represent group 1 and are described as altricial (closed eyes and naked) and nidicolous. Tammars are classified as altricial, nidicolous and transported, spending >270–300 days of the 350 days of lactation in the pouch totally dependent on milk. Also see Table 2 for gestation length in days.

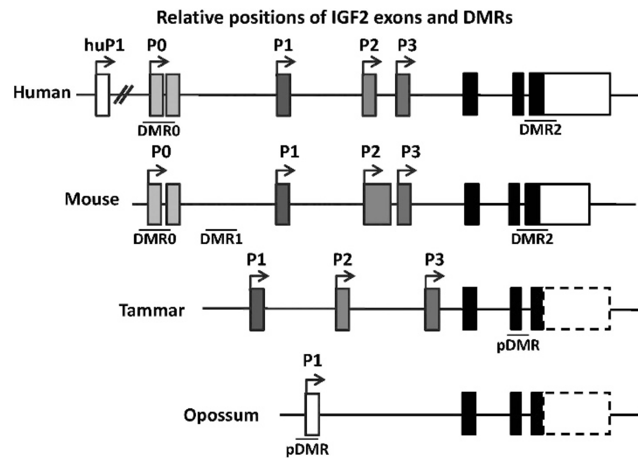

Figure 2 Comparative *IGF2* gene structure. Schematic of human, mouse, tammar and opossum *IGF2* (not to scale). Mouse has four promoters and three DMRs, while human has five promoters. There is no mouse homologue for human P1 (HuP1). The P0 promoters and non-coding exons are homologous to each other as are mouse P1–P3 to human P1–P3. Tammar has three promoters homologous to mouse and human P1–P3 and a putative DMR (pDMR) homologous to mouse and human DMR2. The opossum has one promoter and one non-coding exon and a putative DMR located at the transcription start site. The coding region of mammalian *IGF2* is located in the last three exons (black boxes). Transcription start sites are indicated with turned arrows. Homologous non-coding exons are represented by coloured boxes: P0 (orange), P1 (blue), P2 (Red) and P3 (green); white boxes: non-homologous non-coding exons. A full color version of this figure is available at the *Heredity* journal online.

DIFFERENTIAL METHYLATION AS A CONSERVED MECHANISM OF IMPRINTED GENE REGULATION

The majority of eutherian autosomal imprinted genes are marked by DNA methylation and histone modifications that differ between maternal and paternal alleles. However, the regulatory activities underlying these marks vary among loci. To regulate imprinting, parental-specific epigenetic modifications must be established in the germline, maintained throughout development and then erased before they are re-established in the host germline (reviewed in Saitou and Yamaji, 2010; Hackett *et al.*, 2012; Saitou *et al.*, 2012; Stringer *et al.*, 2013). In eutherians, the majority of epigenetic reprogramming occurs *in utero*, with some *de novo* methylation occurring postpartum in the oocytes (Seisenberger *et al.*, 2012; Tomizawa *et al.*, 2012). In marsupials, germ cells are still proliferating postpartum and do not begin to enter meiosis (females) or mitotic arrest (males) until 25 days after birth (Ullmann *et al.*, 1997; Renfree and Shaw, 2001). Nevertheless, the relative timing and mechanisms of germ cell reprogramming are conserved between eutherians and marsupials, suggesting that these mechanisms evolved well over 160 million years ago (Suzuki *et al.*, 2013).

DMRs are associated with almost all eutherian imprinted gene clusters, but the presence of the DMR is not necessarily the sole epigenetic modification required for monoallelic expression. Other repressive epigenetic marks, such as histone modifications, are likely to have a significant role. In somatic cells, the methylated allele within an imprinting control region (ICR) is associated with repressive histone modifications, including H4 lysine 20 and H3 lysine 9 tri-methylation (H4K20me3 and H3K9me3), whereas the unmethylated allele is enriched for permissive histone modifications, such as H3K4me2 and acetylated H3 (H3ac) (Delaval *et al.*, 2007). However, germline DMRs are the main epigenetic mark distinguishing parental alleles of imprinted genes in the early embryo. Thus far, only three DMRs have been identified in marsupials in the promoters of *PEG10*, *H19* and *IGF2R* (Suzuki *et al.*, 2007; Smits *et al.*, 2008; Das *et al.*, 2012). An additional two putative DMRs have also been identified in *IGF2* and *INS* (Lawton *et al.*, 2008; Stringer *et al.*, 2012b, c). The existence of homologous DMRs in both marsupials and eutherians suggests that, for some genes, genomic imprinting regulated by differential methylation evolved before the divergence of these mammal groups (Suzuki *et al.*, 2007; Smits *et al.*, 2008).

IGF2 was the first imprinted gene identified in eutherians and marsupials and encodes a growth-promoting gene that is maternally silenced in all therian species so far examined (DeChiara *et al.*, 1991; O'Neill *et al.*, 2000). *Igf2* modulates both placental supply and fetal demand of nutrients (Constancia *et al.*, 2002; Reik *et al.*, 2003). Imprinting of *IGF2* and *INS* in mice and humans is regulated by the *H19*/*IGF2* ICR. The ICR is located approximately 2 kb upstream of *H19* and contains several CpG sites and four highly conserved 11-zinc-finger nuclear protein (CTCF) binding sites (Bell and Felsenfeld, 2000; Hark *et al.*, 2000; Kanduri *et al.*, 2000; Szabo *et al.*, 2004). There are several spatially and temporally regulated endoderm and mesoderm enhancers downstream of the *H19* gene (Gabory *et al.*, 2006). The binding of CTCF to the unmethylated maternal ICR blocks the interaction of the enhancers with the *IGF2* promoters and silences the maternal allele. Methylation of the DMR on the paternal allele prevents CTCF protein binding, repressing the *H19* promoter while allowing *IGF2* expression. This ICR and DMR is highly conserved in marsupials, suggesting that DMR regulatory mechanisms arose in the therian ancestor (Smits *et al.*, 2008).

Eutherian *IGF2* has multiple promoters and transcription start sites adjoining distinct non-coding exons: four in rodents and five in humans (Figure 2) (Rotwein and Hall, 1990; Vu and Hoffman, 1994; Monk *et al.*, 2006). The opossum neonate and the adult platypus produce only a single *IGF2* transcript, and only one opossum

non-coding exon has been identified (Killian *et al.*, 2001; Lawton *et al.*, 2008). Interestingly, tammar *IGF2* has three promoters and non-coding exons, orthologous to the eutherian P1–P3. Additionally, a putative tissue-specific DMR, othologous to mouse and human DMR2 (Monk *et al.*, 2006), was identified in the tammar placenta (Figure 2) (Stringer *et al.*, 2012b). Opossum *IGF2* also has a possible DMR, located at the single transcription start site, but it is not orthologous to either the mouse or human DMRs (Lawton *et al.*, 2008). This suggests independent selection for DMR-associated silencing in two disparate lineages (Stringer *et al.*, 2012b).

Human *IGF2R* has a DMR but lacks the parental promoter allele-specific histone modifications that are thought to regulate imprinted expression of mouse Igf2r. Hence, human *IGF2R* is biallelically expressed (Yang *et al.*, 2003; Vu *et al.*, 2004). In marsupials, *IGF2R* is imprinted and possesses a novel DMR not present in eutherian mammals (Das *et al.*, 2012). Interestingly, there is an allele-specific permissive histone modification (H3K4me2) at the IGF2R promoter (Das *et al.*, 2012). Although the repressive mark H3K9me3 is absent, other modifications (for example, H3K27me3, H4K20me3) may be present and function to repress the paternal allele (Delaval *et al.*, 2007; Henckel *et al.*, 2009; Das *et al.*, 2012). Therefore, some DMRs are conserved between marsupials and eutherians at some loci, while at other loci epigenetic mechanisms have clearly diverged between these groups.

IMPRINTING IN THE MAMMARY GLAND

The role of imprinted genes in pre-natal nutrient transfer and placental development is well established (Coan *et al.*, 2005; Constancia *et al.*, 2005; Renfree *et al.*, 2013). However, there is comparatively little information on the possible role of imprinted genes in the provision of post-natal resources and care during lactation. Unlike the placenta, which contains fetal-derived cells with genetic contributions from both of the offspring's parents, the mammary gland is comprised purely of maternal cells. Therefore, only a matrilineal grandparental genetic contribution is regulating post-natal resource allocation.

In mouse mammary epithelial cells, X-chromosome inactivation is not random but is preferentially maternally inactivated (Jiao *et al.*, 2012). Interestingly, the X-linked gene ring finger protein 12 (*Rnf12*), which encodes the ubiquitin ligase Rnf12/RLIM, is a critical survival factor for milk-producing alveolar cells (Jiao *et al.*, 2012). In humans, limited studies of gene expression in the breast have similarly demonstrated monoallelic expression of growth and survival factors in normal breast tissues.

However, during breast cancer, there is mis-regulation of a number of monoallelic genes. Breast cancer is genetically heterogeneous, and a variety of genetic lesions have been identified that tend to accumulate during disease progression. Six genes, all with potential roles in cancer progression, are monoallelically expressed (presumed to be imprinted) in the mammary gland and show mis-expression in various breast cancers, namely, *IGF2*, *H19*, distinct subgroup of the RAS family (*DIRAS3*), mesoderm-specific transcript (*MEST/PEG1*), human *MAS* proto-oncogene and *IGF2R* (Yballe *et al.*, 1996; Oates *et al.*, 1998; Pedersen *et al.*, 1999; Yu *et al.*, 1999).

MEST is monoallelically expressed in normal breast tissue, so it may be imprinted and involved in the regulation of gland growth in humans (Pedersen *et al.*, 1999). In the mouse, *Mest* is imprinted and paternally expressed in a variety of adult tissues, albeit at lower levels than the developing embryo (Reule *et al.*, 1998; Takahashi *et al.*, 2005). *Mest* appears to be involved in the formation of white adipose tissue, including the determination of adipose cell size (Takahashi *et al.*, 2005). Interestingly, white adipose tissue is vital for the mammary gland development (Couldrey *et al.*, 2002). Thus, *Mest* may be required for the development and function of the mammary gland in addition to its role in the hypothalamus. However, *Mest* expression has not yet been investigated in the mouse mammary gland, and the monoalleic expression in the human breast has not yet been attributed to genomic imprinting.

DIRAS3 (*NOEY2/ARHI*), a putative tumour-suppressor gene, is imprinted and paternally expressed in cultured human breast epithelial cells (Yu *et al.*, 1999, 2003). Interestingly, *DIRAS3* deletion (presumably paternally inherited) occurs in a substantial fraction of human breast cancers (Yu *et al.*, 1999). Overexpression of a human *DIRAS3* transgene in mice is associated with a decrease in body size, greatly impaired mammary gland development and lactation, decreased fertility, loss of neurons in the cerebellar cortex and impaired development of the thymus (Xu *et al.*, 2000). *Mas* is a proto-oncogene maternally imprinted in mice in a highly developmental and tissue-specific manner (Villar and Pedersen, 1994). In mice, *Mas* is an important modulating factor in the electrophysiology of the hippocampus and is involved in behavioural pathways in the adult brain (Walther *et al.*, 1998). In humans, *MAS* is biallelically expressed in the fetus (Riesewijk *et al.*, 1996), but monoallelic expression has been detected in normal breast tissue (Miller *et al.*, 1997). This indicates the possible presence of a functional imprint at this locus in humans, but this finding requires further investigation to see if it is truly a result of genomic imprinting.

In the tammar, lactation takes up to 9–10 months while the young increases from a birth weight of 450 mg to 2.5 kg when they are fully weaned (Figure 1). Active pregnancy is <1 month in duration in the tammar, and gastrulation to birth is only about 10 days, so the energy transfer from mother to young is proportionately less than in eutherian mammals (Figure 1; Table 2). However, by the time of weaning and permanent pouch exit, the energy transfer from mother to young is roughly equivalent to that of a sheep to its newborn lamb (Cork and Dove, 1989). There are dramatic changes in milk volume, milk composition and individual milk constituents throughout marsupial lactation (Green, 1984). In contrast, apart from the initial production of colostrum 24–48 h postpartum, the composition of mature eutherian milk changes are not as dynamic and are species-specific (Oftedal and Iverson, 1995). There are three broad phases in marsupial lactation that reflect these changes (Figure 3). Phase 1 is the time during pregnancy when the mammary gland prepares for lactation; Phase 2 (Day 0–200) is the initiation of lactation and production of the early milk, which is characterised by the high carbohydrate–low fat content. During Phase 3 (days 200–350) fat content rises steadily, whereas carbohydrate levels fall (Tyndale-Biscoe and Renfree, 1987). In addition, there are tightly regulated changes in the specific proteins and amino acids during each phase (Renfree *et al.*, 1981; Nicholas, 1988; Trott *et al.*, 2003). Thus if imprinting was maintained to regulate maternal investment and nutrient transfer, the acquisition of imprinted genes in the mammary gland may be more important than those in the placenta to marsupials.

Insulin (*INS*) is a key gene required for carbohydrate metabolism, intra-uterine growth and the establishment of lactation. In mammals, insulin, in addition to cortisol and prolactin, is an absolute requirement for synthesis of milk (Menzies *et al.*, 2010). In the tammar, insulin is a crucial requirement to stimulate milk-protein synthesis and establish lactation (Nicholas *et al.*, 1991; Trott *et al.*, 2002, 2005). In eutherians, *INS* has only been confirmed as imprinted in the yolk sac placenta (Deltour *et al.*, 1995; Moore *et al.*, 2001). In marsupials, *INS* is imprinted in the yolk sac placenta, but the maternal allele is not

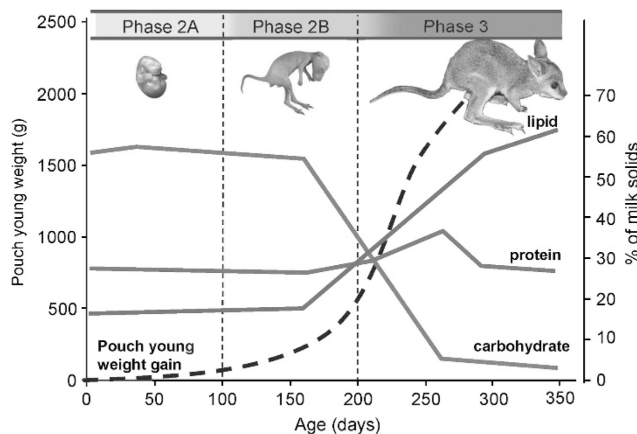

Figure 3 Milk composition and pouch young growth. Changes in protein, carbohydrate and fat content of milk (redrawn from Green, 1984). There are four stages of lactation in the tammar (Tyndale-Biscoe and Renfree, 1987). Phase 1 encompasses the initiation of lactogenesis in late gestation. Phase 2A spans the first 100–125 days of pouch life when the young is permanently attached to the teat, followed by Phase 2B to day 200 postpartum, when the young can relinquish the teat and sucking becomes more intermittent. Phases 2A and 2B are characterised by milk that is high in carbohydrate and low in fat. Phase 3 of lactation (days 200–350) includes the period of rapid growth of the young when it begins to exit the pouch and starts to eat grass, up until weaning. This phase is characterised by low-carbohydrate, high-fat milk. Growth curve data provided by Renfree and Shaw (unpublished data). A full color version of this figure is available at the *Heredity* journal online.

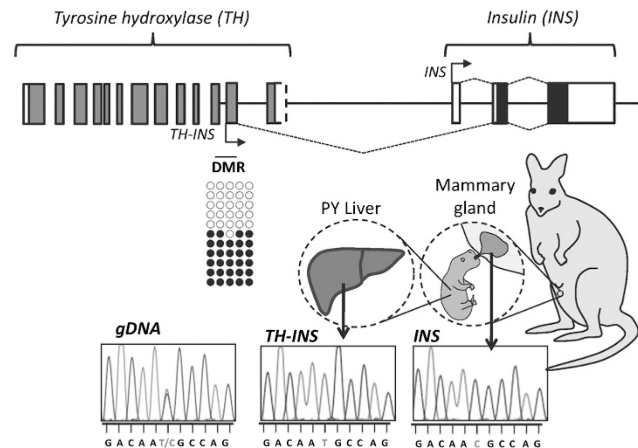

Figure 4 Schematic of predicted tammar *TH* and *INS* genes and the *TH-INS* and *INS* transcripts (not to scale). Predicted coding exons (grey), verified coding exons (black) and non-coding exons (white) are represented by boxes. Transcription start sites identified are indicated by turned arrows. The putative DMR is shown with individual bisulphite sequences underneath: open and closed circles are unmethylated and methylated CpGs, respectively. Each row represents the methylation pattern on a separate DNA fragment from the same sample. Both methylated and unmethylated alleles were present in the liver and mammary gland tissues at the *TH-INS* transcription start site. *TH-INS* and *INS* chromatogram traces (viewed in FinchTV version 5.1) for genomic DNA (gDNA) and complementary DNA (cDNA) derived from the pouch young liver and the adult mammary gland. The single-nucleotide polymorphism identified in the gDNA was used to determine monoallelic expression in the liver and mammary gland.

completely silenced in all individuals (Ager *et al.*, 2007). Consistent with eutherians studies, *INS* is not imprinted in the marsupial pancreas (Ager *et al.*, 2007). Further analysis identified an alternative *INS* transcript expressed in the liver and mammary gland but not in the pancreas (Stringer *et al.*, 2012c). This transcript contained an exon derived from the neighbouring tyrosine hydroxylase gene (*TH*) along with the two coding *INS* exons (Figure 4). Non-transcript-specific analysis identified that *INS* is monoallelically expressed and is likely to be imprinted in the marsupial post-natal liver and mammary gland (Stringer *et al.*, 2012c). Preliminary transcript-specific sequencing data and the identification of a putative DMR at the *TH-INS* transcription start site in both liver and mammary gland suggests that both *TH-INS* and *INS* are imprinted (Stringer *et al.*, 2012c). Although it is unknown whether the *TH-INS* transcript produces a functional protein in the marsupial, a similar chimeric transcript produced in the chicken does (Hernandez-Sanchez *et al.*, 2006). However, it is possible that the *TH-INS* is a non-coding RNA, and transcription may provide an alternative mechanism to regulate *INS* expression. For example, *TH-INS* may be transcribed from the maternal allele and block *INS* transcription. Further analysis of the *TH-INS* and *INS* expression profiles and promoters is required to confirm this hypothesis (Figure 4).

IGF2 is a growth-promoting maternally imprinted gene (paternally expressed) in all therian species studied (DeChiara *et al.*, 1991; Deltour *et al.*, 1995; O'Neill *et al.*, 2000). In the active mammary gland, *IGF2* together with cyclin-D1 acts as a mediator of the prolactin-induced proliferation of mammary epithelial cells during alveolar formation (Brisken *et al.*, 2002). Tammar *IGF2* is imprinted and paternally expressed in the fetal and pouch young liver and has paternally biased biallelic expression in the whole placenta and pouch young brain (Suzuki *et al.*, 2005; Smits *et al.*, 2008). *INS* and *IGF2* imprinting is regulated by the ICR, located between *IGF2* and *H19*, that is conserved in marsupials (Smits *et al.*, 2008). Similar to *INS*, *IGF2* is also monoallelically expressed in the adult mammary gland but not in the adult liver (Stringer *et al.*, 2012b, c). As *H19* is biallelically expressed in the liver (Smits *et al.*, 2008), an alternative mechanism must be regulating *TH-INS* and *INS* monoallelic expression in this tissue. The observation of biallelic expression of *IGF2* in the adult liver and clear monoallelic expression of both *INS* and *IGF2* in the mammary gland strengthens the notion of a link between nutrient transfer and genomic imprinting (Stringer *et al.*, 2012b).

POST-NATALLY IMPRINTED GENES AND THE KINSHIP HYPOTHESIS

Imprinted genes affect both the growth and transport capacity of organs involved in nutrient supply (for example, placenta) and modulate fetal requirements for nutrients, for example, by controlling fetal growth (Reik *et al.*, 2003). Thus, the selective advantage of monoallelically expressing a gene in a population must outweigh the cost of an increased mortality rate. There are many hypotheses to explain the selective advantage that maintains genomic imprinting (Wilkins and Haig, 2003). The kinship (parental conflict) hypothesis is the most widely supported hypothesis, which predicts that parent-specific gene expression may be maintained to exploit the asymmetry in parental resource contribution to a given offspring (Haig and Westoby, 1989; Haig, 2004). In the offspring, expression of paternally inherited resource-acquisition genes are predicted to be modified to increase the growth of his offspring and to promote the extraction of the largest possible quantity of resources from its mother. This is of no fitness cost to the father, but the increase in size, fitness and reproductive success of his offspring increases his genetic fitness. Conversely, maternally expressed genes are predicted to reduce an

individual offspring's resource demand so that she may provide for future offspring to maximise her reproductive fitness. Genomic imprinting is thought to have arisen as a result of this conflict. With regard to *in utero* resource allocation, the placenta is the most obvious site of conflict between parental genomes and is the main site of imprinted gene expression. Additionally, the pre-weaning stage of development is another potential conflict arena and recent studies have identified imprinted genes in the mother and the post-natal offspring that regulate the growth of the offspring.

Gnasxl is an imprinted gene, which is paternally expressed in key areas of the brain that innervate the facial and tongue muscles (Peters *et al.*, 1999; Plagge *et al.*, 2004). Deletion of this gene reduces the sucking behaviour of the newborn pups and results in substantially reduced milk intake and weight gain compared with wild-type littermates (Plagge *et al.*, 2004). *Gnasxl* is therefore a post-natally imprinted gene that supports the kinship hypothesis for imprinting. The paternally inherited *Gnasxl* allele promotes milk intake, while the maternally inherited allele is silenced.

There are many imprinted genes with multiple functions; some of their functions are consistent with the predictions of the kinship hypothesis while others are harder to explain. Both *Mest* and *Peg3* are paternally expressed and enhance fetal growth. In post-natal mice, these genes regulate pup attachment to the teat and sucking behaviour (Li *et al.*, 1999; Curley *et al.*, 2004). However, mutant females lack normal maternal behaviour such as placentophagia, retrieval of pups, nest building and normal suckling behaviour, which reduces survivability of her offspring. Maternal behaviour is not influenced by the offspring's father, and there is no obvious or direct conflict between grandparental genomes over the investment of the mother's resources. Therefore, the functions of *Mest* and *Peg3* regulating maternal behaviour are inconsistent with the original kinship hypothesis. Although, some very minor asymmetries may occur if there is inbreeding in a population and if the degree of matrilineal vs patrilineal relatedness is skewed between a female and her mate (Wilkins and Haig, 2003). However, the selection from this minor asymmetry over maternal care is likely to be very weak and is unlikely to be the main selective force behind the evolution of imprinting at these loci (Wilkins and Haig, 2003).

Recent advances to the kinship theory define conditions for cooperation as well as conflict in mother–offspring relations, and imprinting of genes affecting maternal and communal care could have been driven by intragenomic conflict, distinct from parental conflict (Ubeda and Gardner, 2010, 2011; Haig, 2014). Further large-scale analyses of imprinted gene expression and function in the brain and other organs, such as the mammary gland and placenta, in a variety of social and 'anti-social' species are necessary to validate these predictions.

THE CO-ADAPTATION HYPOTHESES

An alternative hypothesis was proposed by Wolf and Hager (2006), called the maternal–offspring co-adaptation theory for the evolution of genomic imprinting (Wolf and Hager, 2006). They suggested that genes involved in the intimate maternal–offspring interaction are more likely to be maternally expressed as it enhances the genetic integration of these co-adapted traits. These traits are expected to be regulated by genes that are imprinted in the offspring but are biallelically expressed in the mother to increase their genetic relatedness. In support of this hypothesis, cross-fostered mice pups receive more provisioning from foster mothers of their own maternal strain irrespective of their father's strain (Hager and Johnstone, 2003).

The majority of imprinted genes are monoallelically expressed in the placenta. The placenta is a unique organ that not only controls pre-natal transfer of maternal resources but also ensures provision of these resources by regulating maternal food intake, maternal behaviour and metabolism. Through the production of hormones, the placenta primes the mother's brain in preparation for post-natal events to ensure mammary gland activation. Imprinted gene knockout mice result in an altered balance between placental and fetal growth and have highlighted the complex signalling used between the mother, placenta and fetus to optimise nutrient transfer (Godfrey *et al.*, 1998; Angiolini *et al.*, 2011; Kusinski *et al.*, 2011; Burton and Fowden, 2012).

Keverne and Curley (2008) expanded the co-adaptation hypothesis to explain how paternally expressed imprinted genes have been maintained at some loci due to co-adaptation of the maternal hypothalamus and the placenta (Figure 5a) (Keverne, 1995; Constancia *et al.*, 2005; Swaney *et al.*, 2007; Keverne and Curley, 2008). In general, genes that are imprinted in the brain (more specifically, the hypothalamus) and the placenta are under matrilineal control. This means that the mother has silenced her own allele in her offspring, allowing the paternal allele to be expressed. As a male has the potential to sire more offspring than a female, paternal haploid expression of maternally imprinted genes have greater potential for rapid fixation of traits in the population. Such advantages rapidly establish homozygosity of a beneficial allele. When a placental/hypothalamic maternally silenced gene is inherited from the father, both sons and daughters benefit from enhanced placental transfer, as well as the good 'communication' from the placenta to the mother's hypothalamus. When this gene is inherited from the mother, it is silenced, but her offspring benefit from good maternal care, increased maternal feeding and milk let-down through the action of the genes in the maternal hypothalamus. Her sons will produce offspring in the next generation expressing her allele, thereby escalating the co-adaptive advantages to the following generation.

The main concern for this adaption of the co-adaptation hypothesis is the inability to predict the direction of imprinting (Haig, 2014). Monoallelic expression of a maternal allele in the male should be disseminated as quickly as a paternally expressed allele; however, this allele would be silenced in his offspring. Instead, the offspring will express a maternal allele, which will increase the offspring's transcriptional relatedness to its mother. Wolf and Hager (2009) suggest that an advantage of resembling or not resembling a parent could favour the evolution of imprinted gene expression. This may, in part, be due to a transgenerational cis epistasis or the 'green-beard effect' (Haig, 1996, 2014). 'Green beard' refers to a gene that can 'recognise' their duplicate in another individual. In other words, if the mothers' responses (that is, nutrient provisioning, maternal care) are attuned to a particular stimulus provided by the offspring (that is, placental hormones, suckling, calling), then any offspring which inherit the genes that produce the required stimulus would receive more maternal resources than offspring that do not (Haig, 1996). Imprinting may allow the mother to 'selectively' invest more of her resources into offspring whose genome, which includes the paternal genomic contribution, appears to be more similar to her own. Thus the co-adaptation hypothesis may be considered a type of kin selection that provides an explanation for the maintenance of imprinted genes in both the pre- and post-natal mammal. However, as Haig (2014) mentioned, if a gene's effects have a cost (trade-off) to either the parental or offspring fitness then genetic conflict will be present. It is very likely that a combination of the kinship, co-adaptation and phenotypic matching hypotheses resulted in the evolution of

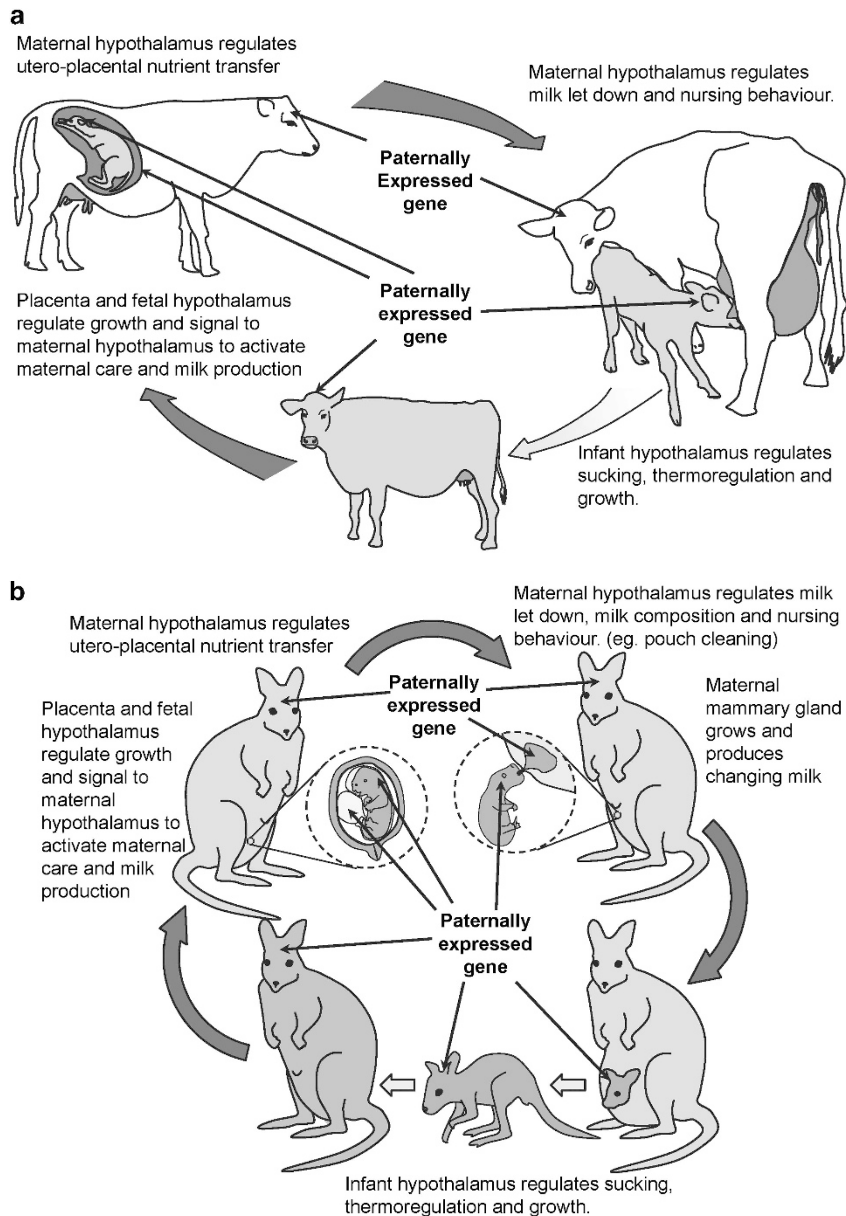

Figure 5 Maternal–infant co-adaptation. (**a**) Pre-natal and post-natal function of paternally expressed (maternally imprinted) genes in the hypothalamus and placenta in eutherians (adapted from Keverne and Curley (2008) and predicted functions (**b**) in the hypothalamus, placenta and mammary gland of marsupials.

imprinting and that it is dependent on gene location and function as to which combination of selective pressures resulted in imprinting. Defining such mechanisms requires an in-depth analysis of tissue-specific imprinting in addition to phylogenetic reconstructions to determine when, where and how imprinting arose in a particular locus.

The mammary gland is a unique mammalian organ that regulates post-natal nutritional transfer by a positive feedback loop with the mother's brain in response to the sucking stimulus. This interaction is similar to that observed between the placenta, fetus and the maternal hypothalamus (Figure 5b). Therefore, imprinting in the mammary gland may be the result of co-adaptation of the maternal and fetal genomes to enhance the genetic integration of the intimate maternal–offspring interactions. If the mammary gland is classed as a 'social organ', then imprinting may have been maintained in this organ due to intra-genomic conflict (Ubeda and Gardner, 2010, 2011; Haig, 2014). However, this selection is dependent on the skewed relatedness between siblings and individuals involved in communal care.

THE EVOLUTION OF IMPRINTING IN THE POST-NATAL MAMMAL

Peg3 provides an excellent example of an imprinted gene that supports the co-adaptation hypothesis for the evolution of genomic imprinting. *Peg3* is involved in maternal care, placental nutrient functions and regulating milk let-down. In addition, *Peg3* confers olfactory advantages enabling male mice to distinguish between females in oestrus and di-oestrus (Keverne, 1995; Li *et al.*, 1999; Curley *et al.*, 2004). *Peg3* also functions in the offspring's

hypothalamus to regulate attachment to the teat and sucking behaviour (Li *et al.*, 1999; Curley *et al.*, 2004). Therefore, this gene would be dispersed quickly through a population when paternally expressed: male mice that are better able to identify oestrous females will sire more offspring that will receive and express this same beneficial allele. The offspring will receive adequate maternal provisioning both pre- and post-natally. Daughters will be genetically predisposed to become good mothers, while sons, like their father, acquire enhanced mating advantages (Keverne and Curley, 2008). Therefore, imprinted expression could be maintained in any organ that functions to regulate the growth and/or growth-influencing behaviour of the individual. The *PEG3* gene contains 13 exons, the last 4 of which originated from the ancestral ZIM2 gene (Kim *et al.*, 2000). *PEG3* transcript encodes an unusual zinc finger protein with 11 widely spaced C2H2-like zinc finger motifs (Kuroiwa *et al.*, 1996). It would be interesting to examine the expression and imprint status of the marsupial *PEG3* gene in the marsupial brain, mammary gland and placenta. However, initial attempts to locate a *PEG3* homologue in the marsupial genome have been confounded by the vast number of genes containing zinc finger motifs (Suzuki *et al.*, 2011b).

IGF2 has an important role in development and brain function. *Igf2* is imprinted in the rodent neonatal granule cells of the cerebellar parenchyma but not in the adult choroid plexus and leptomeninges (DeChiara *et al.*, 1991; Pedone *et al.*, 1994; Hetts *et al.*, 1997). The expression in the granule cells has been associated with a role in cell proliferation and cerebellum weight during fetal and neonatal development (Hetts *et al.*, 1997; Fernandez *et al.*, 2010; Pidsley *et al.*, 2012). In humans, *IGF2* is monoallelically expressed within specific regions of the adult brain while it is biallelic in the fetal brain (Pham *et al.*, 1998). Tammar *IGF2* is imprinted in the placenta, fetal and pouch young liver and pouch young brain (Suzuki *et al.*, 2005; Smits *et al.*, 2008; Stringer *et al.*, 2012b). Therefore, regionally restricted imprinting of *IGF2* in the brain of human, mouse and tammar (Hetts *et al.*, 1997; Suzuki *et al.*, 2005; Smits *et al.*, 2008; Stringer *et al.*, 2012b) suggests that this pattern may have evolved in the therian ancestor. In the brain, IGF2 has been implicated in the regulation of brain morphology, food intake and in memory consolidation and enhancement (Lauterio *et al.*, 1987; Ahmed and Lauterio, 1992; Hetts *et al.*, 1997; Fernandez *et al.*, 2010; Chen *et al.*, 2011; Pidsley *et al.*, 2012). In the adult rat, intracerebroventricular injection of IGF2 decreases food intake while injections into the hippocampus can significantly enhance memory retention. Both of these functions may have attracted imprinted gene expression based on both the kinship and co-adaptation models possibly as a mechanism to regulate gene dosage in the brain. Conditional lineage-specific knockouts of *IGF2* in the rodent brain may identify the specific functions that attracted imprinted expression in certain brain compartments.

MAGE-like 2 gene (*Magel2*; a clock-controlled circadian output gene), like *Mest* and *Peg3*, is a paternally expressed gene in the hypothalamus. Adult mice deficient in *Magel2* have markedly reduced activity, reduced metabolism, increased adiposity after weaning, behavioural problems and impaired male fertility (Bischof *et al.*, 2007; Kozlov *et al.*, 2007). *Magel2*-deficient mice have 50% neonatal mortality with impaired suckling onset behaviour and subsequently impaired feeding (Schaller *et al.*, 2010). Reduced oxytocin was also detected in the hypothalamus of *Magel2* mutant neonates. An injection of oxytocin receptor antagonist replicated the *Magel2* mutant feeding phenotype in wild-type neonates, and a single injection of oxytocin rescued the *Magel2* mutant pup phenotype. Therefore, *Magel2* is required for both milk let-down in the mothers and sucking behaviour in the neonates, which is regulated by paternally expressed imprinted genes in the hypothalamus, supporting the co-adaptation hypothesis.

GRB10 binds to the insulin and IGF1 receptors and possibly inhibits the growth-promoting and glucose homeostasis activities of insulin, IGF1 and IGF2 (Smith *et al.*, 2007; O'Neill *et al.*, 1996). Eutherian *GRB10* transcription is regulated by two promoters, the maternally expressed major-promoter and a second paternally expressed brain-specific promoter. In the mouse, *Grb10* is maternally expressed from the major-promoter in most tissues (Garfield *et al.*, 2011). In humans, the expression of *GRB10* from the major-promoter is biallelic in most tissues except in the placental villus trophoblasts in which it is maternally expressed (Hikichi *et al.*, 2003; Monk *et al.*, 2009). In the brain, *Grb10* is paternally expressed from the brain-specific promoter within the diencephalon, ventral midbrain, medulla oblongata and along the ventral spinal cord (Garfield *et al.*, 2011). However, although GRB10 is expressed in the marsupial brain, they do not have the brain-specific promoter (Stringer *et al.*, 2012a). The expression from the tammar major-promoter is biallelic in a variety of adult and pouch young tissues, including the mammary gland. Therefore, imprinting at this locus evolved in eutherians after the eutherian–marsupial divergence and the emergence of a brain promoter, which may have occurred via the insertion of a parasitic DNA element (Stringer *et al.*, 2012a).

In the mouse, disruption of the maternally expressed *Grb10* transcript results in the overgrowth of both the embryo and the placenta (Charalambous *et al.*, 2003; Charalambous *et al.*, 2010). In the mammary epithelium, activation of signal transducer and activator of transcription 3 and 5 (*STAT3* and *STAT5*, respectively) is sufficient to induce and suppress apoptosis, respectively (Clarkson *et al.*, 2006). As *Grb10* is a transcriptional target of Stat5a (Clarkson *et al.*, 2006), its predicted function is to regulate the survival of mammary epithelial cells, promoting milk protein synthesis or release (Liu *et al.*, 1997). Females who inherited a *Grb10* deletion from their mothers have more pups, but these are smaller and the placental weight is significantly lower (Charalambous *et al.*, 2010). Therefore, *Grb10* may influence reproductive strategy through the allocation of maternal resources such that offspring number is offset against size. As *GRB10* is a pleiotropic gene affecting maternal–offspring interactions, imprinting may have been maintained to increase the adaptive integration between the maternal and offspring genomes (Wolf and Hager, 2006). Further examination of the expression and function of imprinted genes in the eutherian mammary gland is required for a more complete understanding of why genes are imprinted. For example, the human major *GRB10* promoter is thought to be maternally expressed only in the placenta. If this promoter is also imprinted in the human mammary epithelial cells, this would provide further evidence to support the co-adaptation hypothesis. Similarly, further comparisons of imprinted gene structure, expression and regulation between the three mammalian taxa will provide a clearer picture of how and when imprinting evolved at each locus.

CONCLUDING REMARKS

The reliance on a placenta during gestation and on lactation post parturition is a common and defining feature of eutherians, marsupials and monotremes. Viviparity has also evolved in fish and reptiles, especially squamates, that bear live young ones (Blackburn, 2006; Renfree *et al.*, 2013). Therefore, if imprinting was maintained as a result of maternal–infant co-adaptation, then the mammary glands of monotremes and placentas of live-bearing reptiles would be prime targets for future investigation. In marsupials, infants are dependent

on lactation for a much greater proportion of time, so it is perhaps not surprising that some imprinted genes may have been acquired in the mammary gland. Even in mice and humans, it is clear that there are an increasing number of imprinted genes identified in postnatal stages, so perhaps more imprinted genes will be identified in the mammary gland, some of which may be exclusively imprinted in this unique mammalian organ.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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