Evolution of placental structure and function in ruminants

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Summary

The defining feature of ruminant placentation is the fusion of binucleate trophoblast cells with uterine epithelium. It was present in the last common ancestor of ruminants and the fusion process is facilitated by the products of endogenous retroviral genes called syncytins. It provides a mechanism to transfer placental hormones to maternal tissues. One of these hormones is placental lactogen, which likewise was present in the ancestral ruminant. An innovation in the pecoran lineage was the placentome, which enabled the exchange area to be increased compared with the diffuse placenta of chevrotains. Duplication of a hemoglobin gene and evolution of a fetal hemoglobin to improve oxygen transfer probably occurred later. Other gene duplications enabled elaboration of the endocrine repertoire of the placenta and occurred at various stages in the evolution of ruminants. The binucleate trophoblast cells express MHC Class I antigens and can be expected to elicit a maternal immune response. A balance needs to be established whereby the semiallogeneic trophoblast is tolerated whilst maintaining vigilance against infection. Uterine macrophages develop along a pathway where they become immunosuppressive, whereas lymphocytes in the uterine epithelium between placentomes remain primed to respond to pathogens. It cannot be determined how such responses evolved, however, due to the paucity of information on the immune system of the uterus in ruminants and other artiodactyls.

Introduction

The mammalian phylogenetic tree provides a scaffold to view the likely path of placental evolution. Epitheliochorial placentation was present in the common ancestor of Cetartiodactyla (artiodactyls including whales) and three other orders (Vogel 2005; Mess & Carter 2007; Elliot & Crespi 2009). Here the focus will be on the further evolution of placental structure and function in the ruminant lineage. Where relevant, placentation in ruminants will be compared to that in other artiodactyls. In some instances, however, it is instructive to discuss characters that have

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been subject to convergent evolution in ruminants and more distantly related mammals. This applies in particular to the endocrine functions of the placenta, where there are remarkable parallels between ruminants, rodents and primates.

Current understanding of mammalian evolution derives from analysis of molecular and morphological data. For relationships at the ordinal level and above we defer to two recent papers, one based on molecular data at the family level (Meredith *et al*. 2011) and the other strong on morphological characters (O'Leary *et al*. 2013). For relations between families and tribes of ruminants we recommend a recent study (Bibi 2013) that was carefully calibrated by fossil data and based on a mitochondrial genome set (Hassanin *et al*. 2012). For the most part these trees are congruent. For example, all confirm that chevrotains (Tragulidae) are basal to the five families of pecoran ruminants.

Functional morphology and placental exchange

Binucleate trophoblast cells

Ruminants have two types of trophoblast. The first forms an epithelium that is in intimate contact with but does not breach the uterine epithelium. The second is the binucleate trophoblast cell (BNC) that is interspersed between the other cells in this epithelium (Fig. 1) and characterized

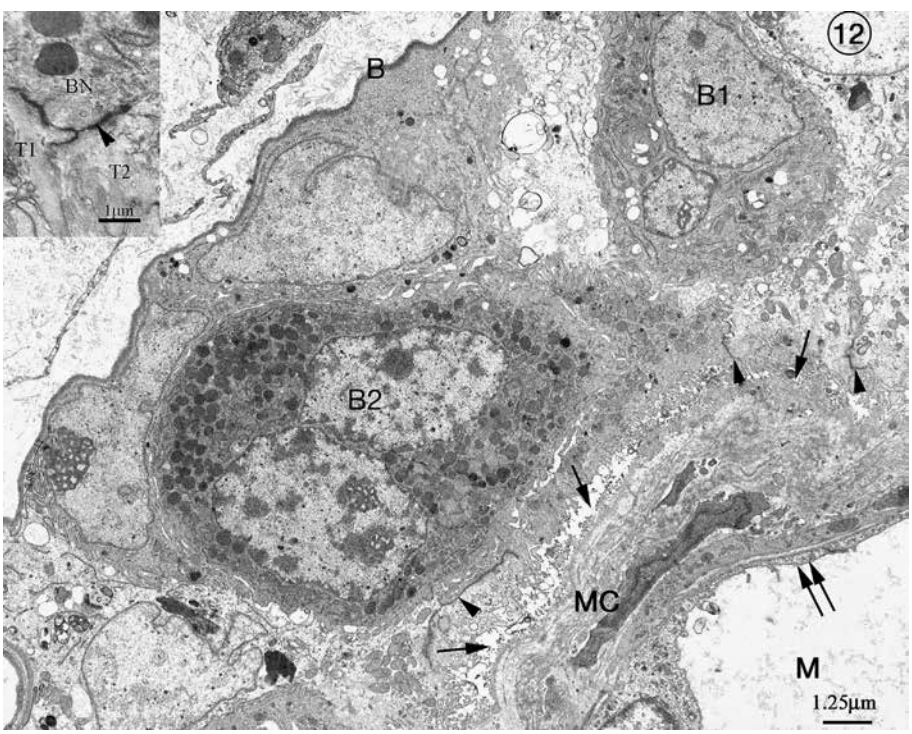

Fig. 1 Early (B1) and late (B2) developmental stages of the binucleate cell in the near term placenta of a chevrotain (*Tragulus* sp.). Note the difference in shape and electron density from the uninucleate trophoblast cells, which are attached to a basement membrane (B). M, maternal capillary; MC, maternal connective tissue; arrow heads, tight junctions; double arrow, maternal capillary endothelium. Reprinted from Wooding et al. (2007) A light and electron microscopical study of the tragulid (mouse deer) placenta. Placenta 28: 1039-1048, © 2007 with permission from Elsevier.

by quite a different repertoire of secreted hormones and cell surface antigen expression. The most remarkable feature of the BNC is its ability to fuse with the uterine epithelium to form either a trinucleate cell, as in the cow, or a more extensive syncytial plaque, as in the sheep and chevrotain (Wooding 1992; Wooding *et al.* 2007). This type of cell is not found in other mammals and seems to have evolved as a means of delivering fetal hormones to the mother.

One step in the evolution of the fusion process has been capture of a retroviral envelope gene, *Syncytin-Rum1*, which is expressed by BNCs in bovine and ovine placentas (Cornelis *et al.* 2013). In retroviruses, the role of the envelope protein is to promote fusion of the viral membrane with the plasma membrane of a host cell. This property is retained by ruminant syncytin and explains how a BNC expressing the protein is able to fuse with a uterine epithelial cell. The gene was shown to be conserved across all higher ruminants (Pecora), but was absent in other orders of mammal (Cornelis *et al.* 2013). One of the most highly conserved parts of the sequence was the immunosuppressive domain.

It has not been possible to identify the *Syncytin-Rum1* gene in chevrotains, where BNCs play the same role in placental development as in higher ruminants, including the formation of syncytial plaques (Wooding *et al.* 2007). It is, of course, possible that the primers used failed to pick up the gene because of sequence divergence in the 50 million years since the ancestor of Tragulidae diverged from that of pecoran ruminants (Cornelis *et al.* 2013).

Expression of a second syncytin-like gene by BNCs has been shown in four species of Bovinae, although not in sheep or goat (Caprinae) (Nakaya *et al.* 2013). It was suggested that this gene, named *Fematrin-1*, could account for the formation of trinucleate cells in bovines as opposed to syncytial plaques in caprines and other ruminants (Nakaya *et al.* 2013). Independent capture of retroviral envelope genes, resulting in multiple syncytins, is known from other orders of mammal (Dupressoir *et al.* 2012).

Another group of retroviruses plays a role in differentiation of BNCs in sheep and goats, although not in cattle. These endogenous retroviruses are related to the pathogenic Jaagsiekte sheep retrovirus (JSRV). Five of them are capable of transcription and all have an envelope gene. They are expressed during blastocyst elongation, when differentiation of BNCs first occurs, and later in BNCs and syncytial plaques (Spencer & Palmarini 2012). A functional role for endogenous JSRVs was demonstrated using antisense oligonucleotides to block JSRV protein production; this inhibited BNC differentiation at the blastocyst stage (Dunlap *et al.* 2006). A role for endogenous JSRVs in cell fusion has yet to be demonstrated, but it is fair to regard them at least as nascent syncytins (Cornelis *et al.* 2013).

Caruncles, cotyledons and placentomes

The most striking feature of pecoran placentation is the presence of multiple placentomes (Andresen 1927). They develop where the chorion overlays the uterine caruncles. The fetal component of a placentome is the cotyledon, but the term frequently is used to refer to the complete structure (Wooding & Burton 2008). The common ancestor of pecorans had a uterus with four rows of caruncles in each horn and a large number of placentomes ("polycotyledonary placentation"). Reduction to a single row of caruncles and fewer placentomes ("oligocotyledonary placentation") was a later development in the lineages of deer and musk deer (Klisch & Mess 2007). Meanwhile, greater complexity in the internal folding of the fetal villi evolved in reindeeer (Hamilton *et al.* 1960) and among the bovids (Hradecky 1986; Hradecky *et al.* 1988). It is reflected in the increased complexity of vessel branching on the fetal and maternal sides of the placentome. In the sheep, capillary branching increases across gestation and doubtless supports a steady increase in the supply of oxygen and nutrients to the fetus (Reynolds *et al.* 2010).

Placental exchange

Sheep have long been the model of choice in fetal physiology (Carter 2011). Therefore a great deal is known about placental transfer of respiratory gases (Longo 1987; Carter 1989), sugars (Hay, Jr. *et al*. 1990) and amino acids (Battaglia & Meschia 1978) in ruminants.

One factor promoting placental oxygen transfer is the greater oxygen affinity of fetal hemoglobin. In catarrhine primates this was achieved by duplication of the *HBG* gene with HBG-T2 serving as the beta chain in fetal hemoglobin. Ruminants have lost the *HBG* gene (Opazo *et al*. 2008) Instead there have been two rounds of duplication of the *HBB* gene. This example of convergent evolution is all the more interesting because a distinct fetal hemoglobin does not occur in any non-ruminant family of Cetartiodactyla. The first duplication of *HBB* was present in the common ancestor of goats, sheep and cattle (Schimenti & Duncan 1984). It likely occurred early in the ruminant lineage since a fetal hemoglobin is known to be present in a cervid, the white-tailed deer (*Odocoileus virginianus*) (Kitchen & Brett 1974). A second duplication yielding four sets of globin genes occurred in the lineage of sheep and goat (Lingrel *et al*. 1985; Townes *et al*. 1984). Thus HBB-2 serves as the beta-chain in the fetal hemoglobin of cattle and HBB-T3 in that of sheep and goat.

Placental transfer of sugars is facilitated by the transporters GLUT1 and GLUT3. In sheep GLUT1 is expressed on the basal membranes of the trophoblast and the maternal syncytium, whereas GLUT3 is localized to the apical surface of the trophoblast (Wooding *et al*. 2005a). The same pattern occurs in the red deer (*Cervus elephas*) and goat (Wooding *et al*. 2005a). These transporters play a similar part in the diffuse placenta of *Tragulus*, but there is the additional detail that GLUT1 protein is associated with granules within the binucleate cells. This raises the interesting possibility that transporter molecules are delivered from the fetal to the maternal side (Wooding *et al*. 2014).

A substantial amount of glucose is converted in the placenta to fructose, which is found at high concentrations in the fetal plasma of ruminants, pigs and whales (Cetartiodactyla) as well as horses (Perissodactyla) (Goodwin 1956) and by inference in their common ancestor. In the fetal pig (a non-ruminant) fructose is metabolized via the hexosamine pathway (Kim *et al*. 2012).

Whilst epitheliochorial placentation does not constitute a barrier to transfer of oxygen and nutrients, special arrangements are required for the transfer of some minerals. The uptake by specialized trophoblast of uterine gland secretions, cell debris or maternal red blood cells is known as histotrophic nutrition. Many ruminants have hemophagous regions within the cotyledons designed for endocytosis and digestion of red cells to supply the fetus with iron (Burton 1982). Iron may also be supplied in uterine gland secretions in the form of uteroferrin as recently described for the water buffalo (*Bubalus bubalis*) (Pereira *et al*. 2009). The glandular secretions, often referred to as uterine milk, are absorbed by areolar trophoblast above the gland openings. The areolae seem also to be important for calcium transport as the areolar trophoblast has particularly strong expression of the 9 kilodalton calcium-binding protein (9-CBP) in both *Tragulus* and the sheep. In addition, 9-CBP is expressed by uninucleate trophoblast cells in the diffuse placenta of *Tragulus* and the interplacentomal areas of sheep, whereas it is absent from the sheep placentome (Wooding *et al*. 1996; Wooding *et al*. 2014).

Endocrinology

Ruminant placentas secrete an impressive array of hormones (Table 1). Some of these act on the uterine glands. In sheep, where this has best been studied, the glands mature under the influence of maternal steroids and the sequential action of interferon-tau (IFN-τ), ovine

placental lactogen and ovine growth hormone from the trophoblast (Noel *et al.* 2003). The placenta synthesizes steroid hormones and a wide range of cytokines, but the emphasis here will be on peptide hormones. Each of these has arisen through gene duplication, although at different points in evolution.

Table 1 Peptide hormones secreted by the placenta of ruminants

Hormone	Distribution	Derivation	Evolution
Interferon-τ (*IFNT*)	Pecoran ruminants	Interferon-ω (*IFNW*)	Gene duplication
Placental lactogens and prolactin-like proteins	All ruminants	Prolactin (*PRL*)	Tandem duplication in cattle, sheep and goat to yield multiple genes
Placental growth hormone	Sheep and goat	Growth hormone (*GH*)	Gene duplication
Pregnancy-associated glycoproteins	Artiodactyls	An aspartic proteinase	Ancient and recent genes, the latter from a second round of gene duplication in ruminants

Interferons

IFNT, the gene coding for IFN-τ, arose in the pecoran lineage through duplication of *IFNW*, which codes for interferon-omega (IFN-ω) (Roberts *et al.* 1998; Roberts *et al.* 2003). IFN-τ is secreted by trophoblast at the blastocyst stage and its principal function is maintenance of pregnancy. It acts by binding to receptors in the endometrium and suppressing pulsatile secretion of the luteolytic factor prostaglandin $F_{2\alpha}$ (Han *et al.* 1997). This is achieved indirectly by silencing estrogen receptor alpha, thereby reducing expression of oxytocin receptor and thus the oxytocin-induced pulsatility of prostaglandin $F_{2\alpha}$ secretion (Fleming *et al.* 2006).

Placental lactogens

Placental lactogen evolved following duplication of the prolactin gene (*PRL*) (Forsyth & Wallis 2002). The gene is expressed in the binucleate trophoblast cells. Based on an immunostaining protocol, it is present in *Tragulus*; thus placental lactogen is common to all ruminants (Wooding *et al.* 1992). However, while some ruminants have a single placental lactogen, there has been tandem duplication in cattle, which have 13 *PRL*-like genes (Larson *et al.* 2006; Ushizawa *et al.* 2005), and in the sheep and goat (Ushizawa *et al.* 2007b; Ushizawa *et al.* 2007a). Tandem duplication of the *PRL* gene as the basis for placental hormones is also known from murid rodents (Forsyth & Wallis 2002). This is yet another interesting example of convergent evolution.

Placental lactogen binds to receptors in the uterine glands (homodimers of prolactin receptor and heterodimers of prolactin and growth hormone receptors) to stimulate hyperplasia (Noel *et al.* 2003). In addition, it plays a subsidiary role in maintenance of the corpus luteum (Buttle 1978). It appears not to play any significant role in mammary gland development or lactation (Bassett *et al.* 1998). Although ovine placental lactogen is an agonist at the prolactin receptor, it acts as an antagonist at the growth hormone receptor (Herman *et al.* 1999), causing speculation that the placental lactogens of ruminants are players in maternal-fetal conflict over partition of nutrient resources (Haig 2008).

The other PRL-like genes in cattle, sheep and goat code for prolactin-like proteins (PRPs). Most are expressed in binucleate cells but their receptor has not been identified and their function remains obscure.

Placental growth hormone

In sheep and goat there is duplication of the growth hormone gene (*GH*) (Wallis *et al.* 1998). Clearly this has occurred in the lineage of subfamily Caprinae, since there is only a single *GH* gene in cattle (Gootwine 2004). Ovine placenta expresses the pituitary *GH* gene as well as that of placental growth hormone (*GH2-Z*) (Lacroix *et al.* 1996; Wallis *et al.* 1998). Ovine placental growth hormone is secreted for a limited period (35-70 days gestation) (Lacroix *et al.* 1996), but acts synergistically with placental lactogen to promote the uterine gland hypertrophy within this time frame (Noel *et al.* 2003).

Pregnancy-associated glycoproteins

Pregnancy-associated glycoproteins (PAGs) make up a family of genes that appeared in Cetartiodactyla and continued to evolve in ruminants (Szafranska *et al.* 2006; Hughes *et al.* 2000). PAGs belong to a wider family of aspartic peptidases and have undergone two rounds of gene duplication. The products of the first round, called "ancient PAGs," generally retain the active site (Brandt *et al.* 2007). In pig and cow, they are expressed at the microvillous junction between the uterine epithelium and trophoblast (Wooding *et al.* 2005b). Here they may function as linking molecules and play a role in fetal-maternal anchorage.

The second round of duplication was restricted to the ruminant lineage. In cattle, many of the resultant gene products lack the active site of the enzyme, although this is retained in two recent PAGs from the white-tailed deer (Brandt *et al.* 2007). In general, the recent PAGs are expressed predominantly on the surface of binucleate cells, although deer PAG-3 also is expressed by the mononucleate trophoblast cells (Brandt *et al.* 2007; Wooding *et al.* 2005b). Gene duplication is often associated with the assumption of new functions, and it has been suggested that the PAGs expressed on binucleate cells engage in immunological camouflage and facilitate maternal tolerance of this invasive type of trophoblast (Wooding *et al.* 2005b).

Immunology

The placenta is a semi-allogeneic transplant and some of the antigens expressed on the surface of trophoblast are paternal in origin. Yet the placenta is not rejected by the maternal immune system. One advantage of epitheliochorial placentation may be that trophoblast encounters only the uterine epithelium. Thus, in relation to the immune system, the placenta has a status comparable to that of a commensal organism in the gut (Moffett & Loke 2006). This potential advantage is sacrificed in ruminants because BNCs express MHA Class I antigens (Bainbridge *et al.* 2001) and are in close contact with maternal tissues. There needs to be some degree of immunosuppression, therefore, but this requires a delicate balance as vigilance against infection must be maintained.

The implications for the immune system are difficult to dissect from an evolutionary standpoint because so few species of ruminants and non-ruminant artiodactyls have been looked into. Indeed, information is sparse even for domesticated species. Regulatory T-cells and natural killer cells play a prominent role in the endometrium and decidua of rodents, primates and possibly horses (De Mestre A. *et al.* 2010), but no recent and reliable information is available on their presence or absence in the ruminant endometrium. $CD4^+$ T-cells, $CD21^+$ B cells and $CD14^+$ macrophages are found in the subepithelial layer of non-pregnant cows and their number and distribution is unchanged during blastocyst elongation in early pregnancy (Leung *et al.* 2000).

Immune cells tend to be excluded from the placentomes following implantation, but this has not been studied systematically. There has been greater focus on intraepithelial T-cells and macrophages, which are localized mainly to the interplacentomal regions.

Intraepithelial T-cells

The ovine uterus has a sizeable population of intraepithelial lymphocytes. From around 50 days of gestation, most of the intraepithelial lymphocytes in the interplacentomal regions are γδT cell receptor positive (γδTCR$^+$) cells (Meeusen *et al*. 1993; Fox *et al*. 2010). These are large, granular lymphocytes whose granules contain perforin and granulysin (Fox *et al*. 2010). They are fully differentiated and may play a defensive role toward invading pathogens. Interestingly, γδTCR$^+$ cells, which are large granular lymphocytes, are largely excluded from the placentomes in both sheep and cow (Lee *et al*. 1997; Gogolin-Ewens *et al*. 1989). In contrast, large granular lymphocytes occur within the placentomes of deer (Cervidae). Indeed, in a study comprising six species of deer (Lee *et al*. 1995), large granular lymphocytes were closely associated with degranulating trinucleate cells.

Macrophages and dendritic cells

The endometrial stroma of ruminants contains macrophages. These have been studied most closely in bovine pregnancy. These immune cells are abundant in the interplacentomal regions but occur in lesser numbers within the placentomes (Oliveira & Hansen 2009). Moreover, macrophages in these two locations differ in expression of CD11b and major histocompatibility complex (MHC) class II markers (Oliveira & Hansen 2009).

There is a fundamental difference between the transcriptomes of circulating and endometrial macrophages (Oliveira *et al*. 2010). Evidence suggests that bovine endometrial macrophages differentiate along the M2 pathway, which would endow them with immunosuppressive properties.

Dendritic cells accumulate in the endometrial stroma early in bovine pregnancy. Like macrophages, they have been ascribed an immunomodulatory role (Mansouri-Attia *et al*. 2012).

Synthesis and conclusions

The sequence of events in evolution of ruminant placentation is shown in Table 2. It starts with the evolution of epitheliochorial placentation in the common ancestor of the four orders constituting the clade Fereuungulata. Increased placental conversion of glucose to fructose likely arose in the common ancestor of artiodactyls (including ruminants) and perissodactyls (horses and their kin).

The defining feature of ruminant placentation is the binucleate cell and its ability to fuse with uterine epithelial cells to form a syncytium or a trinucleate cell. BNCs can be dated to the last common ancestor of the chevrotains and pecoran ruminants (Table 2). It seems likely that the fusion process was aided by an endogenous retroviral gene. This may have been an antecedent of *Syncytin-Rum1* found in pecorans or represent an independent gene capture. Multiple independent captures of retroviral envelope genes are known from other taxa (Dupressoir *et al*. 2012). Indeed there is a second syncytin, *Fematrin-1*, that appears in the lineage of the bovine subfamily (Nakaya *et al*. 2013) whilst multiple endogenous *RJSV* genes occur in the caprine subfamily.

Table 2 Timeline of placental evolution in ruminants, showing the appearance of distinct characters in various taxonomic clades.

Taxonomic clade	Branching point (mya)	Geological period	Character	Comments
Fereuungulata	82.0	Cretaceous	Epitheliochorial placentation	Reversion to endotheliochorial state in carnivores
Euungulata	66.5	Cretaceous	Placental conversion of glucose to fructose	
Cetartiodactyla	65.4	Paleocene	Pregnancy-associated glycoproteins	
Ruminantia	40.3	Eocene	Binucleate trophoblast cells and fusion with uterine epithelium	
			Placental lactogens	Further elaborated in Bovidae
Pecora	20.4	Early Miocene	Placentomes	Number reduced in Cervidae and Moschidae
			Syncytin gene (*Syncytin-Rum1*)	
			Interferon-tau gene (*IFNT*)	
Bovidae	16.2	Early Miocene	Duplication of *HBB* gene for high affinity fetal hemoglobin	May have evolved earlier since a fetal hemoglobin has been reported in Cervidae
Bovinae	11.0	Middle Miocene	Syncytin gene (*Fematrin-1*)	
Caprinae	10.1	Middle Miocene	Endogenous JSRV retroviral genes	
			Placental growth hormone	

Approximate dates (million years ago, mya) above the family level are taken from Meredith et al. (Meredith *et al.* 2011) and for families and subfamilies from Bibi (Bibi 2013). Fereuungulata comprises the orders Perissodactyla, Pholidota, Carnivora and Cetartiodactyla.

From a functional standpoint, fusion of BNCs and epithelial cells facilitates delivery to the maternal tissues of cytokines and hormones made in the BNC. Placental lactogen is the best known signal. It is already present in chevrotains and acts to enhance uterine gland secretion and nourishment of the embryo with histotroph. PAGs, which had evolved in the artiodactyl lineage, underwent further elaboration in the ruminants, where many are expressed by BNCs.

The next major event in ruminants was the evolution of placentomes. The common ancestor of extant pecorans had four rows of caruncles in each uterine horn and a large number of placentomes. Placentomes greatly increase the exchange area of the placenta (Baur 1977) and may have been a necessary adaptation to ensure an adequate supply of substrates to the fetus (Klisch & Mess 2007). Reduction to a single row of caruncles and far fewer but larger placentomes occurred later in the lineages of deer (Cervidae) and musk deer (Moschidae).

Another innovation in pecorans was duplication of the *IFNW* gene to yield *IFNT* and secretion of interferon-τ by the trophectoderm of the elongating blastocyst. IFN-τ binds to receptors in the endometrium and suppresses pulsatile secretion of the luteolytic factor $PGF_{2\alpha}$ (Han *et al.* 1997; Fleming *et al.* 2006). It is pertinent to ask how this is achieved in chevrotains, but very little is known about their reproductive biology (Kusuda *et al.* 2013). Maybe another cytokine performs

a similar function, perhaps even interferon-ω. Alternatively, the luteotrophic action of placental lactogen could be sufficient to support luteal function as shown for the hypophysectomized goat (Buttle 1978).

Bovidae is the largest ruminant order by far with >140 species (Wilson & Reeder 2005). Apart from further elaboration of prolactin-like proteins and PAGs, this family is distinguished by duplication of the *HBB* gene yielding the beta globin component ("gamma chain") of fetal hemoglobin. Innovations at the subfamily level include *Fematrin-1* in bovines and en*RJSV*s and placental growth hormone in sheep and goats (Table 2).

It would be fascinating to follow the response of the maternal immune system to such innovations as BNCs and placentomes. Unfortunately too little information is available for ruminants and non-ruminants alike to place this in an evolutionary context.

One can only guess at the selection pressures driving placental evolution. Recognition that epitheliochorial placentation evolved from a more invasive type is of recent date (Vogel 2005; Mess & Carter 2007) and the perceived advantage of isolating the fetal allograft from the maternal immune system (Moffett & Loke 2006) is difficult to substantiate. This is particularly the case because the most successful groups have abandoned a strict separation of maternal and fetal tissues. In horses, trophoblast girdle cells invade the uterus to form hormone-secreting endometrial cups and elicit an immune response (De Mestre A. *et al.* 2010). In ruminants BNCs fuse with uterine epithelial cells as a means to secreting hormones to the maternal system. For mammals as a whole there are numerous instances of convergent evolution (Carter 2012). One example is the evolution in ruminants and primates of fetal haemoglobins with high oxygen affinity. Another is the emergence of different mechanisms to maintain luteal function exemplified in ruminants by IFNT. Care must be taken in extrapolating from ruminants to phylogenetically distant groups, such as primates, and their validity as animal models assessed on a case to case basis. Elsewhere (Carter 2007) it is argued that the sheep is a useful model for studies of placental gas transfer. In contrast, placental lactogens evolved convergently in ruminants and primates through duplication of different genes and it is hazardous to extrapolate from one to the other. Comparative studies are of greatest utility when adequately informed by phylogenetics.

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