

The evolution of fetal membranes and placentation in carnivores and ungulates (Ferungulata)

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Abstract

Molecular phylogenetics has made a substantial contribution to our understanding of the relationships between mammalian orders and has generated trees that can be used to examine the evolution of anatomical and physiological traits. We here summarize findings on fetal membranes and placentation in Ferungulata, a clade comprising carnivores, pangolins, and even- and odd-toed ungulates. Their early ontogeny shows several conserved traits such as superficial attachment of the blastocyst, amnion formation by folding, a large allantoic sac and a temporary yolk sac placenta. In contrast, several characters of the chorioallantoic placenta are derived, including the diffuse and cotyledonary placental types in ungulates and zonary placenta in carnivores, specializations of the interhaemal barrier, the presence of areolae or haemophagous regions and lack of stromal decidual cells. Ungulates produce large amounts of placental proteins including placental lactogens and pregnancy-associated glycoproteins. Evolutionary innovations of the placental system may contribute to the high diversity of lifestyles within Ferungulata and be linked to the evolution of highly precocial offspring in ungulates.

Keywords: areolae, Carnivora, Cetartiodactyla, chorioallantoic placenta, decidua, Eutheria, evolution, interhaemal barrier, Perissodactyla, phylogenetic systematics, Placentalia, trophoblast, yolk sac.

Introduction

Molecular phylogenetics and phylogenomics have given us a good understanding of the relationships between various orders of mammal and the sequence in which those orders evolved (Meredith *et al.*, 2011). With the mammalian tree as a guide, it becomes meaningful to examine the evolution of anatomical and physiological traits. Several studies have done this for the placenta, reaching the conclusion that epitheliochorial placentation is a derived trait and not, as had been supposed, a primitive one (Vogel, 2005; Mess and Carter, 2006; Wildman *et al.*, 2006; Elliot and Crespi, 2009). There have been relatively few studies of other aspects of gestation such as blastocyst attachment and fetal membrane development. This will be a focus of the present paper, which reviews results obtained by plotting characters on the mammalian tree (Mess and Carter, 2006) or by integrating them in a grand analysis of molecular and morphological data (O'Leary *et al.*,

2013). Metabolic and endocrine functions of the placenta have not been the subject of a rigid phylogenetic analysis. However, there are some interesting innovations in Ferungulata that deserve mention.

The mammalian tree

Most mammals of interest to veterinarians are part of a clade with a common ancestor referred to as Ferungulata (Fig. 1). There are two sister groups within this clade (Beck *et al.*, 2006; Hou *et al.*, 2009). The first comprises the pangolins (Pholidota) and carnivores (Carnivora). The other encompasses cetaceans plus even-toed ungulates (Cetartiodactyla) and odd-toed ungulates (Perissodactyla). Inclusion of whales and dolphins with the even-toed ungulates is a relatively new development, but well supported by molecular and fossil data (Spaulding *et al.*, 2009).

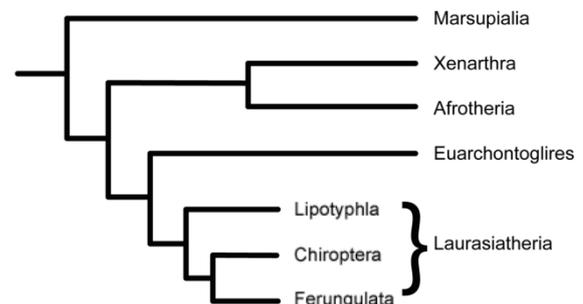


Figure 1. Simplified mammalian tree shows the four major superordinal clades and the position of Ferungulata within Laurasiatheria. Tree topology follows Douady *et al.* (2002) as used for our analysis of the evolution of fetal membrane and placental characters (Mess and Carter, 2006).

Radiation of the placentals gave rise to four superorders named Laurasiatheria, Euarchontoglires, Afrotheria and Xenarthra (Meredith *et al.*, 2011). Ferungulata is placed in Laurasiatheria together with bats (Chiroptera) and hedgehogs, shrews and moles (Lipotyphla), although not all studies support separating Ferungulata from Chiroptera (Meredith *et al.*, 2011; O'Leary *et al.*, 2013). Some of the orders once included in Ferungulata (Simpson, 1945) are now part of Afrotheria; these include the armadillo, elephants, sirenians and hyraxes.

To describe evolution of reproductive characters in a broader sense, we shall refer to them as conserved (plesiomorphic) or derived (apomorphic) character conditions (Hennig, 1966) compared to the

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most recent common ancestor of crown placentals, i.e. living species and their ancestors (Placentalia; McKenna and Bell, 1997). Following the practice recommended by other authors, we shall refer, rather less accurately, to the common ancestor of eutherians.

Fetal membranes

Phylogenetically, the yolk sac is the oldest of the fetal membranes. Amnion, allantois and chorion appeared with the cleidoc egg of reptiles, a major step in vertebrate evolution (Ferner and Mess, 2011). All of these membranes can participate in placentation (Starck, 1959; Mossman, 1987; Wooding and Burton, 2008). The fetal membranes of the sea otter (Fig. 2A; Sinha and Mossman, 1966) illustrate the point. The outermost

membrane is the trophoblast of the chorion. Early in gestation the yolk sac and chorion form a choriovitelline placenta. Later the allantoic sac expands and together with chorion forms a chorioallantoic placenta. There is in addition an exocoelom lined by extraembryonic mesoderm and, where it abuts the uterus, by chorion. The figure denotes a transitional phase in carnivores; the yolk sac is later displaced from the chorion due to expansion of the exocoelom and allantoic sac.

This scheme is applicable throughout Ferungulata, although with minor variations; the ruminant yolk sac, for example, does not form a choriovitelline placenta. As has recently been reviewed (Carter, 2015; Carter and Enders, 2016), development of the fetal membranes proceeds quite differently in other mammals, including human and mouse.

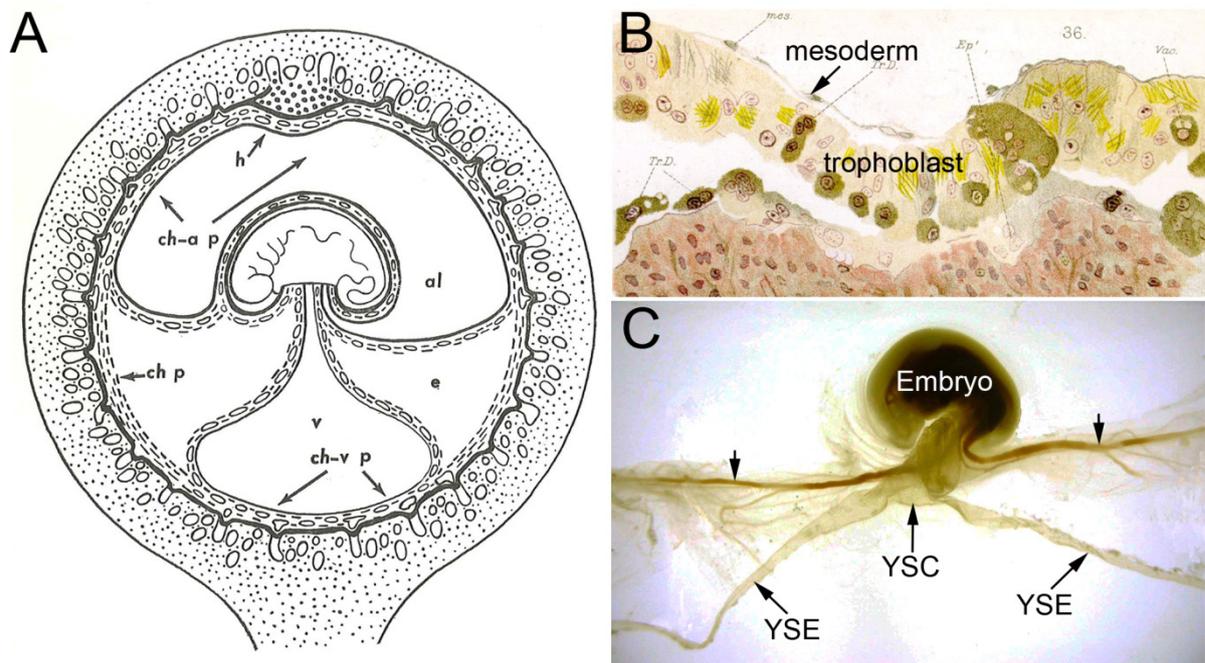


Figure 2. Fetal membranes during early development of a carnivore and a ruminant. A. Membranes of the sea otter (*Enhydra lutis*) during the transition from choriovitelline (ch-v p) to chorioallantoic (ch-a p) placentation; note the paraplacental chorion (ch p). Reprinted with permission from Sinha and Mossman (1966). B. Pre-implantation blastocyst of the sheep (*Ovis aries*); at 20 days gestation. The trophoblast is lined throughout by extra-embryonic mesoderm and the yolk sac is no longer attached. Reprinted from Assheton (1906). C. The bovine yolk sac at 20 days of gestation comprises a central portion (YSC) and two extended arms (YSE); allantoic vessels (arrows) are seen extending from the embryo. Courtesy of Dr. Antonio Assis Neto.

Implantation

An early event in gestation is attachment of the blastocyst to the uterine wall. In most mammals this occurs at the antimesometrial surface. As shown in Fig. 3A, there was a change in the lineage of Ferungulata and Chiroptera to initial attachment on the mesometrial side (Mess and Carter, 2006). Interestingly, this was reversed in the lineage of carnivores and pangolins, but conserved in the ungulate lineage.

Of greater significance for subsequent

development of the membranes is whether the blastocyst remains superficially attached or becomes enclosed in the endometrium either during nidation or subsequently (primary and secondary interstitial implantation). Three studies have now shown that superficial attachment is the conserved or plesiomorphic state of eutherian mammals (Mess and Carter, 2006; O'Leary *et al.*, 2013; McGowen *et al.*, 2014). This state is present in all members of Ferungulata studied to date, whereas interstitial implantation occurs in higher primates and many rodents.

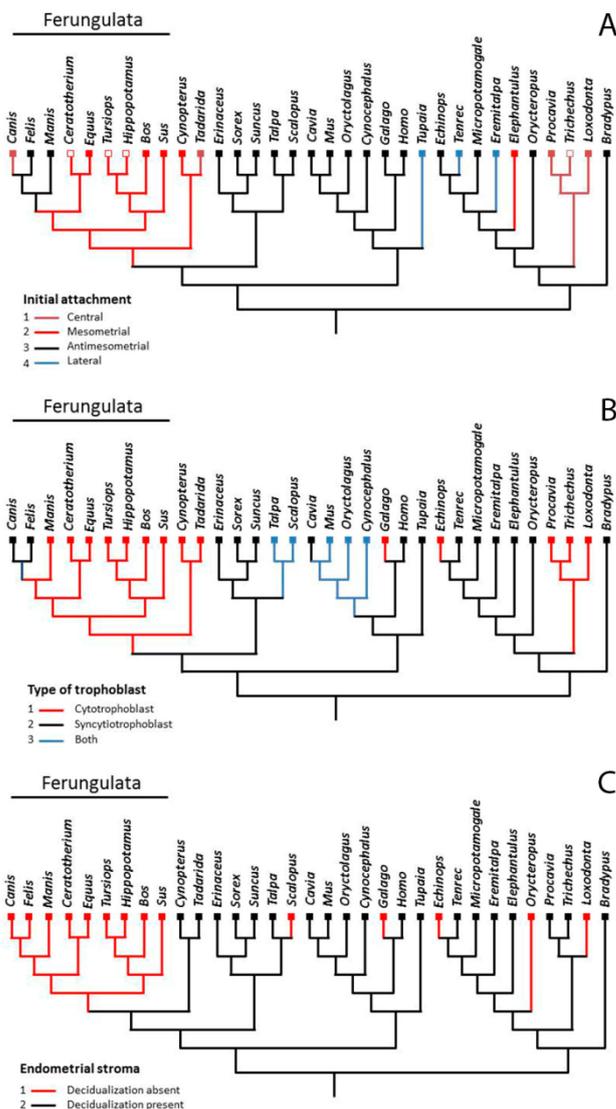


Figure 3. Evolution of implantation and placentation in eutherian mammals. In these phylogenetic trees, Carnivora is represented by dog (*Canis*) and cat (*Felis*); Pholidota by a pangolin (*Manis*); Perissodactyla by rhinoceros (*Ceratotherium*) and horse (*Equus*); and Cetartiodactyla by bottlenose dolphin (*Tursiops*), hippopotamus (*Hippopotamus*), cow (*Bos*) and pig (*Sus*). A. Initial attachment of the blastocyst. The conserved or plesiomorphic condition for eutherians was implantation at the antimesometrial surface of the uterus. This changed to mesometrial implantation in the common lineage of Ferungulata and Chiroptera [represented by a fruit bat (*Cynopterus*) and a free-tailed bat (*Tadarida*)]. B. Type of trophoblast in the interhaemal barrier at term. The plesiomorphic condition for eutherians was a layer of syncytiotrophoblast. This changed to cytotrophoblast in the common lineage of Ferungulata and Chiroptera. There was a reversal in the carnivore lineage. C. Endometrial stroma. The plesiomorphic condition for eutherians was decidualization of the endometrium. This was lost in the lineage of Ferungulata, which do not have decidual stroma cells. Data derived from Mess and Carter (2006).

Amniogenesis

In marsupials and most orders of eutherians, the amnion develops as folds of the extraembryonic somatopleure at the cephalic end and sides of the early embryo. The folds meet and fuse dorsal to the embryo. Several mammals, however, lack amniotic folds or have only rudimentary ones. In the rhesus monkey (Enders *et al.*, 1986) and human (Shahbazi *et al.*, 2016), the amnion forms by cavitation as a space appears amidst a group of polarized cells in the epiblast. In the mouse, a proamnion appears in the centre of the epiblast, but the true amnion is formed from the amniochorionic fold (Dobrevá *et al.*, 2010). However, all members of Ferungulata retain the plesiomorphic state, which is amnion formation by folding (Mess and Carter, 2006).

Yolk sac and choriovitelline placentation

The blastocyst cavity is converted to a primary yolk sac when extraembryonic endoderm (the hypoblast) spreads around the inside of the trophoblast. Subsequently, during expansion of the

exocoelom and allantois, the endoderm is partly or wholly detached and becomes lined by extraembryonic mesoderm. Where it remains in contact with trophoblast as a two-layered structure it is referred to as the bilaminar omphalopleure. Despite lacking blood vessels, the bilaminar omphalopleure serves as a major route for absorption of uterine secretions in, for example, marsupials (Padykula and Taylor, 1982) and the pre-implantation horse embryo (Allen, 2001). Where the attached yolk sac is supplied with mesoderm, generally with vitelline blood vessels, it is referred to as a trilaminar omphalopleure. Development of a trilaminar omphalopleure at some stage of gestation is regarded as plesiomorphic, i.e. it most likely was present in the common ancestor of eutherians (O'Leary *et al.*, 2013) as well as that of Ferungulata (Mess and Carter, 2006). A choriovitelline placenta is present when the vascular part of the yolk sac is in contact with the trophoblast (Fig. 2A).

Although the choriovitelline placenta is transitional in carnivores, the yolk sac persists after detachment from the trophoblast and is found as a T-shaped structure right up to term (Lee *et al.*, 1983;



Miglino *et al.*, 2006). In pangolin there is likewise a prominent choriovitelline placenta early in gestation that later is displaced by the expanding allantois and subsequently dwindles (De Lange, 1933).

During the first three weeks of equine pregnancy, the conceptus develops within an acellular capsule. At this time the non-vascular part of the yolk sac (bilaminar omphalopleure) plays an essential role in the absorption of nutrients derived from uterine gland secretions (Allen, 2001; Waelchli and Betteridge, 2013). Following dissolution of the capsule, tufts of trophoblast emanating from the non-vascular yolk sac protrude into the mouths of the endometrial glands (Allen, 2001). There is, however, a short-lived vascular portion (trilaminar omphalopleure) that forms a choriovitelline placenta (Enders and Liu, 1991; Allen, 2001). The region between the regressing yolk sac and developing allantois is the site at which the chorionic girdle forms.

There is some variation in Cetartiodactyla. In the pig, the yolk sac rapidly loses contact with the trophoblast, but the detached yolk sac persists within the exocoelom through the first third of gestation (Heuser, 1927). The endoderm forms cell columns and these cells have abundant rough endoplasmic reticulum as well as large mitochondria, suggesting their main function is protein synthesis (Tiedemann and Minuth, 1980). In contrast, in ruminants, the vascular yolk sac does not make contact with the trophoblast (Fig. 2B), but remains in the exocoelom as a free floating structure with two extended arms (Fig. 2C; Assheton, 1906; Russe *et al.*, 1992; Assis Neto *et al.*, 2010). The situation in ruminants is comparable to that in bats, where the yolk sac can form a gland-like structure (Carter and Mess, 2008). Similarly, a free-floating yolk sac is found in human and other higher primates, but as a rule it disappears later in gestation (Enders and King, 1993).

Finally rodents, lagomorphs (e.g. rabbits), colugos and most insectivores undergo a process of yolk sac inversion. Where this is complete it results in the endoderm facing the uterine cavity. The inverted yolk sac of rodents is an important conduit for histotrophe and maternal-fetal transfer of antibodies (King and Enders, 1993; Carter, 2015).

Allantois

The size of the allantoic sac varies across mammals, but a medium to large allantoic sac is plesiomorphic and conserved in all members of Ferungulata. In contrast, the allantoic sac is absent in higher primates and many rodents, the connection between embryo and chorioallantoic placenta being supplied by an allantoic stalk (Carter *et al.*, 2015).

Amniotic and allantoic fluids

The volume of fluid in the fetal membranes is substantial. In cattle at 225 days gestation, for example, there are about 2.5 liters of amniotic and 6-9 liters of allantoic fluid (Bongso and Basrur, 1976). The origin and function of these fluids is not fully known despite comprehensive studies in swine (Goldstein *et al.*, 1980),

cattle (Wintour *et al.*, 1986) and sheep (Wintour *et al.*, 1994; Gilbert, 1999). Most of the water is acquired through maternal-fetal transfer with only a minor contribution from water produced during fetal oxidative metabolism (Meschia, 1955). Fetal urine drains to the allantoic sac through the urachus and from about mid-gestation to the amniotic sac via the urethra. There is a further contribution to amniotic fluid of fetal lung liquid. Amniotic fluid is swallowed by the fetus and absorbed from the gastrointestinal tract. In steady state, these processes represent recirculation of fluid. Quite a substantial volume of urine is secreted; about 400 ml to each compartment in sheep (Gilbert, 1999). Maintenance of constant volumes can be explained only by uptake to the fetal vessels of the amniochorion and chorioallantois, known as the intramembranous pathway (Gilbert, 1999). This may be mediated by aquaporin 9, a water channel that is expressed in amniotic and allantoic epithelia, but not in the chorion or placentomes (Wang *et al.*, 2005).

The composition of amniotic and allantoic fluids differs substantially from that of the fetal urine and this can be explained only by the rapid movement of solutes in and out of these fluids presumably via the intramembranous pathway (Gilbert, 1999).

In reptiles as well as mammals, the amnion provides a fluid environment for embryonic development. The function of the allantoic sac is less well understood (Wintour *et al.*, 1994). In species with epitheliochorial placentation, initial expansion of the allantoic fluid may be an important mechanism for bringing the the chorioallantois in contact with the uterine epithelium as suggested by Bazer and colleagues (Goldstein *et al.*, 1980). In addition amniotic and allantoic fluids may act as nutrient reservoirs (Goldstein *et al.*, 1980).

Chorioallantoic placenta

There is considerable variation among mammals in the shape, internal structure (interdigitation) and interhaemal barrier of the placenta (Carter and Enders, 2004; Wooding and Burton, 2008; Chavatte-Palmer and Tarrade, 2016). In all three respects members of Ferungulata deviate from what is thought to be the conserved state in eutherians.

Placental shape

Diffuse placentation, where there is extensive contact between the chorioallantoic membrane and uterine wall, is found in pangolins, perissodactyls and most members of Cetartiodactyla including cetaceans, hippopotamus, pigs, peccaries and camels. Equids differ somewhat in possessing microcotyledons. Among ruminants, chevrotains also have diffuse placentation, whereas pecorans have a varying number of placentomes (Klisch and Mess, 2007). All carnivores have zonary placentation (Miglino *et al.*, 2006).

The conserved state of eutherians is the discoid placenta (Mess and Carter, 2006; Wildman *et al.*, 2006; Elliot and Crespi, 2009) as familiar from human and



mouse. This shape does not occur among Ferungulata. Diffuse placentation likely emerged in the common ancestor of Ferungulata with zonary placentation representing a further evolutionary step in the lineage of carnivores.

Interdigitation

Interdigitation between fetal villi and maternal trabeculae is usually associated with diffuse placentation. It reaches greater complexity in cotyledonary placentas (Hradecky *et al.*, 1988; Klisch and Mess, 2007) and zonary placentas (Capellini, 2012). In higher primates the fetal villi do not interdigitate with maternal trabeculae but are surrounded by maternal blood. The third type of interdigitation, the placental labyrinth, is plesiomorphic and is conserved, for example, in rodents and lagomorphs (Mess and Carter, 2006; Wildman *et al.*, 2006; Elliot and Crespi, 2009).

Interhaemal barrier

Consideration of the tissue layers separating fetal and maternal blood suggests three principal types of interhaemal barrier. In epitheliochorial placentation, the uterine epithelium remains largely intact (Carter and Enders, 2013). This type occurs throughout Ferungulata with the exception of carnivores. Otherwise it is seen only in strepsirrhine primates (lemurs and lorises). For pecoran ruminants, where uterine epithelial cells undergo fusion with binucleate trophoblast cells (see below), the term synepitheliochorial is sometimes preferred (Wooding and Burton, 2008).

In endotheliochorial placentation, the trophoblast is in contact with the maternal capillary endothelium, which often is hypertrophied (Enders and Carter, 2012b). This type of interhaemal barrier occurs in all carnivores with the exception of hyaenas. Finally, in haemochorial placentas the trophoblast is directly in contact with the maternal blood (Enders and Carter, 2012a). Among Ferungulata this has been observed only in hyaenas (Enders *et al.*, 2006). Endotheliochorial and haemochorial states are together referred to as invasive placentation (Carter and Pijnenborg, 2010; Carter *et al.*, 2015).

Vogel was the first to show the epitheliochorial condition is a derived type (Vogel, 2005). That has been confirmed numerous times though there is disagreement about whether the plesiomorphic state is endotheliochorial (Mess and Carter, 2006; Martin, 2008) or haemochorial (Wildman *et al.*, 2006; Elliot and Crespi, 2009).

Trophoblast of the placental barrier

Placentas differ as to whether the trophoblast present in the interhaemal barrier at term is syncytiotrophoblast, cytotrophoblast (cellular) or both (Fig. 3B). Our analysis (Mess and Carter, 2006) suggests that the common ancestor of eutherians had just syncytiotrophoblast. This is of interest in the context of placental evolution as the propensity for

trophoblast cells to fuse and form multinucleate masses is linked to the expression of syncytins. They are the product of endogenous retroviral envelope genes, which have been acquired independently in several lineages (Dupressoir *et al.*, 2012). Because syncytiotrophoblast often accomplishes the initial penetration of the uterine epithelium, it has even been suggested that capture of a retroviral gene was an essential first step in the evolution of placentation (Lavialle *et al.*, 2013).

As Fig. 3B suggests, replacement of syncytiotrophoblast by cytotrophoblast occurred in the common ancestor of Ferungulata and Chiroptera. There were, however, subsequent reversals. The first occurred in the lineage of ruminants. A remarkable feature of their placenta is the ability of a binucleate trophoblast cell to fuse with a uterine epithelial cell to form a hybrid that either is a trinucleate cell as in the cow or a syncytium as in the sheep or the chevrotain (Wooding, 1992; Klisch *et al.*, 1999; Wooding *et al.*, 2007). Tellingly, ruminants have captured a retroviral envelope gene (*Syncytin-Rum1*) and binucleate cells thereby express a syncytin that is able to promote cell fusion *in vitro* (Cornelis *et al.*, 2013). There is evidence of an additional but similar gene in bovines (Nakaya *et al.*, 2013).

The second exception is in carnivores, where there is an apparent reversal towards the presence of syncytiotrophoblast in the barrier (Fig. 3B). Carnivores have captured a syncytin gene (*Syncytin-Car1*) that is expressed in the placenta of dog and cat (Cornelis *et al.*, 2012). This gene is conserved throughout Carnivora with evidence for purifying selection and conservation of fusogenic activity. It does not occur in the pangolins (Pholidota), which retain the epitheliochorial state (Cornelis *et al.*, 2012).

Areolae and haemophagous organs

Columnar trophoblast cells, polarized with their endocytic and lysosomal systems oriented for ingestion, are widespread among mammals. Generally this trophoblast is heterophagous, ingesting uterine secretions, cellular debris or maternal erythrocytes (Enders and Carter, 2006). Histotrophic nutrition assumes greater importance where placentation is epitheliochorial, since haemotrophic nutrition is constrained by the absence of direct access by trophoblast to maternal blood. It is particularly important to have alternate routes for fetal acquisition of iron and copper (McArdle *et al.*, 2008; Carter, 2012).

Two types of adaptation occur in ungulates. The first is the areola, in which columnar trophoblast is arranged above gland openings. In pig (Renegar *et al.*, 1982) and horse (Fig. 4A; Wooding *et al.*, 2000), endometrial glands secrete the iron-rich glycoprotein uteroferrin, which is taken up by the polarized trophoblast of the areolae. Recently, uptake of uteroferrin was also described in the water buffalo (*Bubalus bubalis*; Pereira *et al.*, 2009). Although the uteroferrin gene (tartrate-resistant acid phosphatase type 5) is found in other mammals, its central role in fetal iron supply likely emerged in the ungulate lineage. In sheep and other ruminants (Burton *et al.*, 1976; Burton,

1982), there are, in addition, haemophagous regions designed to ingest maternal erythrocytes and extract iron from the haemoglobin (Fig. 4B).

Similar adaptations occur in carnivores. Thus, the polar zone of the chorioallantois of the cat exhibits pinocytotic activity (Leiser and Enders, 1980a), whilst the remainder of the paraplacenta is haemophagous (Leiser and Enders, 1980b). A prominent haemophagous organ occupies a central, antimesometrial position in the sea otter and other

mustelids, in skunks, and in raccoons and allies (reviewed in Carter and Enders, 2016); these three families share a common ancestor (Eizirik *et al.*, 2010). Even the haemochorial placenta of hyaenas has haemophagous areas (Fig. 4C). According to De Lange, the pangolin placenta lacks specializations such as areolae (De Lange, 1933). There is copious uterine gland secretion, however, and columnar trophoblast similar in appearance to areolar trophoblast (Fig. 4D; Enders and Carter, 2006).

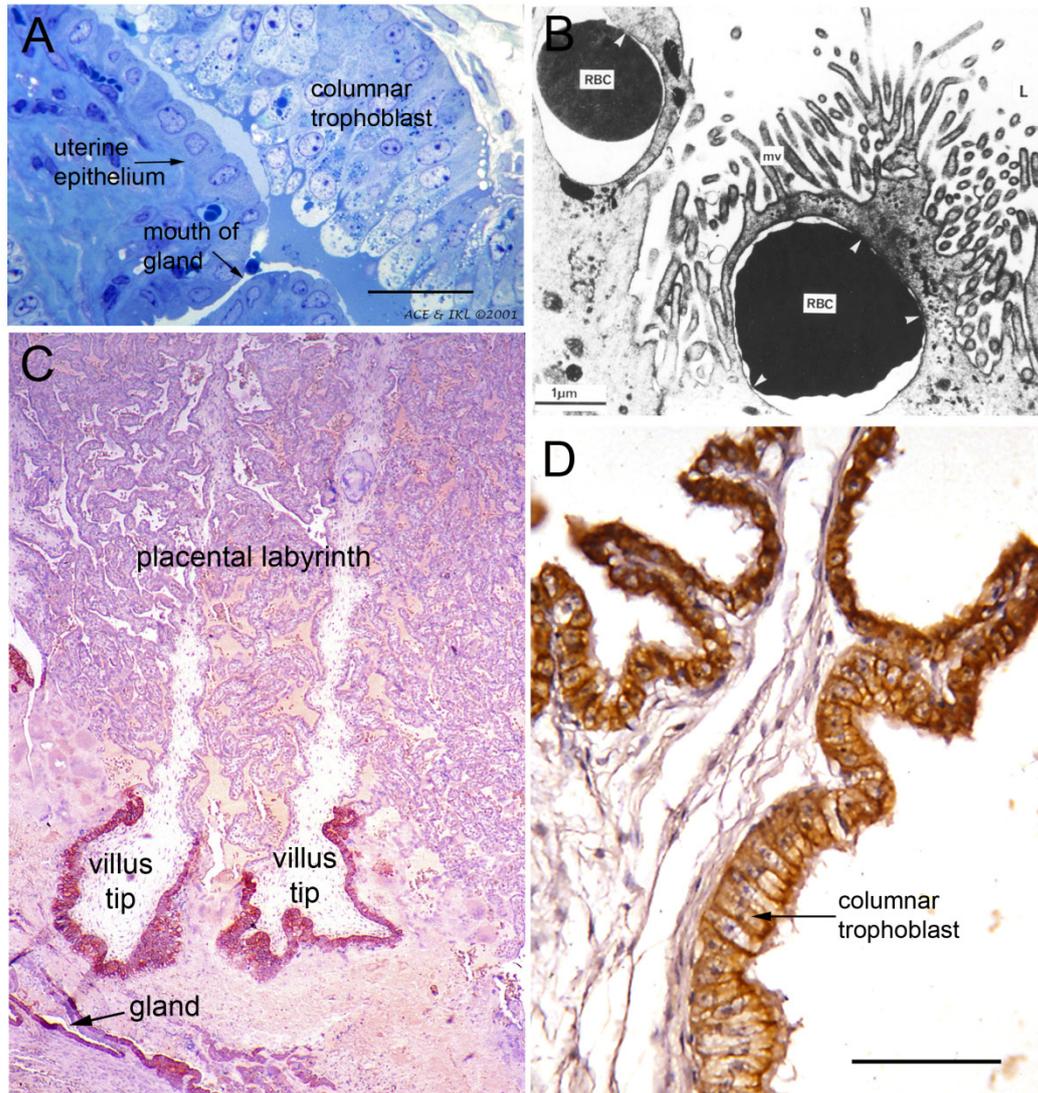


Figure 4. Columnar trophoblast is found in most placentas of carnivores and ungulates. A. Areola of the horse (*Equus caballus*) at 47 days of gestation. Note the columnar trophoblast above the opening of a uterine gland. Reprinted with permission from Carter and Enders (Carter and Enders, 2013). B. Haemophagous region of the cotyledon from a sheep (*Ovis aries*) at 98 days gestation. Two red blood cells (RBC) are enclosed within vacuoles in the apical cytoplasm of columnar trophoblast cells. Scale bar: 1 μ m. Reproduced with permission from Burton *et al.* (1976). C. Intraplacental haemophagous region of a spotted hyaena (*Crocuta crocuta*). The columnar trophoblast at the tip of the villus stains for cytokeratin and is heterophagous. The uterine glands below also stain for cytokeratin. Reprinted with permission from Carter and Enders (2016). D. Fetal villus of a pangolin (*Manis* sp.) with trophoblast stained for cytokeratin. Note the columnar trophoblast cells. The trophoblast has pulled away from the endometrial folds (not in field). Reprinted with permission from Enders and Carter (2006).

Decidua

There has been interest of late in the evolution

of uterine responses to pregnancy, particularly the differentiation of endometrial stromal fibroblasts into decidual stromal cells. We had found that a decidua was



present in the eutherian common ancestor, but was lost in the lineage of Ferungulata (Fig. 3C; Mess and Carter, 2006). Recent studies support the view that evolution of decidua occurred in the eutherian stem lineage coincidental with the appearance of invasive placentation (Wagner *et al.*, 2014; Chavan *et al.*, 2016). It was possible to define a set of transcription factor interactions that were needed to regulate decidual gene expression (Wagner *et al.*, 2014). Moreover, by analyzing the literature and reviewing archival material, this group came to the conclusion that decidua formation was important in the peri-implantation period; they gave several examples, including armadillo and tenrec, where decidual stromal cells were lost later in gestation. It was suggested that the acquisition of additional endocrine and immune functions by decidual stromal cells, including a role in pregnancy maintenance, first occurred in the lineage of Euarchontoglires (Chavan *et al.*, 2016).

Significantly, their analysis proposed a limited role for decidual stroma cells in laurasiatherian species outside Ferungulata, notably the European mole (*Talpa europaea*), the false vampire bat (*Megaderma lyra*) and the disc-winged bat (*Thyroptera tricolor*; Chavan *et al.*, 2016). The short-lived function of decidua in these orders (Chiroptera and Lipotyphla) was seen to be part of the maternal response to invasive placentation. In this light the disappearance of stromal decidual cells in Ferungulata could be linked to the evolution of non-invasive placentation.

Metabolic and endocrine functions of the placenta

Sugar metabolism

Fructose occurs at high concentrations in the

fetal plasma of ruminants, pigs, whales (Cetartiodactyla) and horses (Perissodactyla), but not that of dog, cat or ferret (Carnivora) or any other mammal (Goodwin, 1956). Experiments in sheep (Alexander *et al.*, 1955) and pig (White *et al.*, 1979) have established that a substantial amount of glucose is converted to fructose in the placenta. In a porcine trophectoderm cell line, Bazer and colleagues (Kim *et al.*, 2012) could show that fructose stimulates cell proliferation by activation of the MTOR signalling pathway and synthesis of hyaluronic acid through the hexosamine metabolic pathway. Placenta does not utilize fructose as a primary source of metabolic energy, but it is possible that the fetus metabolizes fructose through the pentose shunt (Bazer *et al.*, 2014). Either way it is clear that placental conversion of glucose to fructose arose in the ungulate lineage.

Placental protein and peptide hormones

Placenta-specific hormones arise through gene duplication (Table 1). Convergent evolution may occur as in the emergence of chorionic gonadotrophins by duplication of the gene for the β -subunit of luteinizing hormone, which happened in the lineages of equids and anthropoid primates (Carter, 2012). Equine chorionic gonadotropin is secreted by the trophoblast of the endometrial cups (Wooding *et al.*, 2001) and acts as a luteotrophic factor. Another example of a peptide that maintains corpus luteum function is interferon- τ (IFN- τ), which arose through duplication of the IFN- ω gene (Roberts *et al.*, 2003). In pecoran ruminants, IFN- τ is secreted by the trophectoderm of the blastocyst and acts by binding to receptors in the endometrium and suppressing pulsatile secretion of the luteolytic factor prostaglandin F 2α (Fleming *et al.*, 2006).

Table 1. Evolution of placental protein and peptide hormones in ungulates and carnivores.

Hormone	Derivation	Distribution	Remarks
Equine chorionic gonadotropin (eCG)	Luteinizing hormone β -subunit	Equids	
Interferon- τ (IFNT)	Interferon- ω (IFNW)	Pecoran ruminants	
Placental lactogens and prolactin-like proteins	Prolactin (PRL)	All ruminants	
Placental growth hormone	Growth hormone (GH)	Sheep and goat	
Pregnancy-associated glycoproteins (PAGs)	An aspartic proteinase	Cetartiodactyla.	Ancient and recent genes, the latter from a second round of gene duplication in ruminants
Pregnancy-specific glycoproteins (PSGs)	Carcinoembryonic antigen cell adhesion molecule (CEACAM) family	Five in the horse	
Relaxin-like factor	Relaxin	Described in the dog	Expressed in genital tract and placenta

The placental lactogens and prolactin-like proteins of ruminants have arisen by tandem duplication of the prolactin gene (PRL). While some ruminants have a single placental lactogen, cattle have as many as 13 PRL-like genes (Ushizawa *et al.*, 2007). Ovine placental lactogen (oPL) binds both to growth hormone (GH) and PRL receptors in the uterine glands thereby

stimulating gland hyperplasia and secretion of histotrophe (Noel *et al.*, 2003). There is sequential action on the glands of IFN- τ , oPL and ovine placental GH (Noel *et al.*, 2003). Placental lactogen may play a subsidiary role in corpus luteum maintenance (Buttle, 1978).

Placental expression of pregnancy-associated



glycoproteins (PAGs) occurs throughout Cetartiodactyla (Wallace *et al.*, 2015). As with placental lactogens, the number of *PAG* genes varies and is highest in cattle (Telugu *et al.*, 2009). PAGs belong to a wider family of aspartic proteinases and have undergone two rounds of gene duplication. The “ancient” PAGs retain the active site. In pig and cow they are expressed at the microvillous junction between uterine epithelium and trophoblast (Wooding *et al.*, 2005). They may function as linking molecules and contribute to fetal-maternal anchorage. The second round of duplication occurred in ruminants and many of the resultant proteins lack the active site. They are expressed mainly at the surface of binucleate cells and have been ascribed immunomodulatory functions (Wooding *et al.*, 2005; Wallace *et al.*, 2015). A PAG-like gene has been characterized in equids (horse and mountain zebra, *Equus zebra*), where it is expressed in the trophectoderm of the blastocyst, including girdle cells, and trophoblast of the term placenta (Xie *et al.*, 1997; Green *et al.*, 1999). The promotor of equine PAG resembles that of a bovine PAG gene (Green *et al.*, 1999). A related protein has been cloned from the cat (Green *et al.*, 1999; Hughes *et al.*, 2003), but its expression pattern is unknown. The equine and feline variants are not glycosylated, however, and are now referred to as pepsin-F (Wallace *et al.*, 2015). It is thought that PAGs proper evolved in the Cetartiodactyla lineage and were derived from an ancestral pepsin-F gene that in turn originated from the pepsin-A gene (Wallace *et al.*, 2015).

Pregnancy-specific glycoproteins (PSGs) are placental hormones found in primates and rodents, where they appear to be associated with invasive placentation (Carter, 2012). They are not found in dogs or cattle, yet seven PSG-like genes have now been identified in the horse (Kammerer and Zimmermann, 2010; Aleksic *et al.*, 2016). Five of them are expressed in trophoblast, notably in the invasive trophoblast of the chorionic girdle and endometrial cups (Aleksic *et al.*, 2016). Evolution of these placental proteins in the horse has been linked conceptually to the transition from a non-invasive epitheliochorial placenta to one with an invasive component in the shape of girdle cells (Kammerer and Zimmermann, 2010; Aleksic *et al.*, 2016). Work currently in progress should clarify whether equine PSGs exert immunomodulatory properties similar to those of rodents and primates. If so this is a remarkable instance of convergent evolution.

In contrast to the ungulates, placental peptide hormones do not play a prominent role in carnivores, where reproductive endocrinology is best understood for the dog (Kowalewski, 2012). There is a canine relaxin-like factor expressed in the male and female reproductive tracts and by trophoblast (Klonisch *et al.*, 2001). No information is available about placental hormones in pangolins.

Placental evolution

The early development and fetal membranes of

ungulates, carnivores and pangolins (Ferungulata) presents an interesting mix of conserved and derived characters. Conserved traits include superficial implantation, amniogenesis by folding, formation of a temporary choriovitelline placenta and retention of a large allantoic sac. There is one derived character, initial implantation at the mesometrial rather than antimesometrial side, for which we have no interpretation.

The placenta of ungulates and pangolins exhibits many derived characters, including its shape, mode of interdigitation and interhaemal barrier. Perhaps linked to this suite of characters is the absence of decidua formation (Chavan *et al.*, 2016). Reversals occur mainly among the carnivores, especially with respect to the interhaemal barrier, which is endotheliochorial in most carnivores and haemochorial in hyaenas.

Ungulates in particular are large mammals with long gestation periods and give birth to a single or few well-developed (precocial) young. These are also derived traits as the ancestral mammal is thought to have been small, had a short gestation period, large litters and poorly developed (altricial) neonates (O'Leary *et al.*, 2013). The change in reproductive strategy necessarily implies heavy maternal investment in each pregnancy. As discussed in greater detail elsewhere (Carter and Enders, 2013), the evolution of epitheliochorial placentation may have been driven by the need to prime the immune system to combat uterine infection rather, as in more invasive placentation, to suppress immune responses in favour of allowing trophoblast invasion. Analogous reasoning has been presented elsewhere (Moffett and Loke, 2006), and the epitheliochorial placenta compared to a commensal residing in the gut. Certainly there is a resemblance to gut in that a large number of intraepithelial lymphocytes can be observed in the uterus of horse and ruminants (Enders and Liu, 1991; Lee *et al.*, 1997; Fox and Meeusen, 1999; Fox *et al.*, 2010).

There was a major reversal in the lineage of carnivores towards shorter gestations, larger litters and more altricial neonates. It is feasible that this change in strategy placed different constraints on the immune system allowing return to a more invasive placental type.

The mammalian tree is a work in progress

There remain uncertainties about the order of branching of the mammalian tree. The ancestor of placental mammals emerged in the Cretaceous, but there was rapid diversification close to the Cretaceous-Palaeogene boundary when mammals occupied ecospace vacated through extinction of the non-avian dinosaurs (O'Leary *et al.*, 2013; Phillips, 2016). Because even the major clades emerged within a short time span, it has proven difficult to root the mammalian tree (Nishihara *et al.*, 2009; Dos *et al.*, 2012). Much evidence favours the configuration in the tree we used for our analysis (Douady *et al.*, 2002), where Afrotheria and Xenarthra are sister taxa and sister to all other



mammals (Waters *et al.*, 2007; Meredith *et al.*, 2011; Dos *et al.*, 2012), but there are dissenting views (Beck *et al.*, 2006; McCormack *et al.*, 2012). In respect of the character states found in the eutherian common ancestor, our findings are in general agreement with the analyses of O'Leary *et al.*, although they placed Xenarthra near the root of the tree (O'Leary *et al.*, 2013).

There is also some disagreement about the relation between Chiroptera and Ferungulata as well as the branch order within the latter clade (Nishihara *et al.*, 2006; Nery *et al.*, 2012), but analyses of retroposed elements (Kriegs *et al.*, 2006) and phylogenomic data (Hou *et al.*, 2009) support the configuration on which our analysis is based. Some character transformations occur in the common ancestor of Ferungulata and Chiroptera and our interpretation would not be affected by a difference in branch order. All major studies on placentation have assumed Ferungulata (Mess and Carter, 2006; Wildman *et al.*, 2006; Elliot and Crespi, 2009), but placental characters would be less easy to resolve if Chiroptera were included in the mix, as there is great variation even among the families of bats (Carter and Mess, 2008).

Conclusion

The eutherian taxon Ferungulata, which is well supported by molecular phylogenies, comprises clades with very diverse lifestyles such as large ungulates, carnivores and pangolins. Nevertheless, they share important traits of early ontogeny and fetal membrane development that have been conserved from the most recent common ancestor of extant eutherians. In contrast, their placentation is characterized by derived conditions. In ungulates, for instance, evolution of their precocial reproductive strategy is associated with the establishment of an epitheliochorial placenta and loss of decidual stromal cells. Although carnivores have reverted to the altricial reproductive strategy of the common ancestor of extant eutherians, they likewise possess features of placentation that represent derived conditions within the group, i.e. a zonary placenta with syncytial trophoblast in the barrier. Thus, evolutionary innovations of the placental system have contributed to the diversity of lifestyles within Ferungulata. Understanding the evolution of this system allows better comprehension of its plasticity. It may help to address problems associated with implantation or endometrial dysfunction in production animals and wild species.

Conflict of interest statement

The authors declare that they have no conflicting interests.

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