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Glycosylation and immunocytochemistry of binucleate cells in pronghorn (*Antilocapra americana*, Antilocapridae) show features of both Giraffidae and Bovidae

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ABSTRACT

Although the pronghorn (*Antilocapra americana*) resembles an antelope, its nearest relatives are the giraffe and okapi. In this study we have examined the placentae of 6 pronghorns using lectin- and immunocytochemistry to identify giraffid and bovid features. Binucleate cells (BNC) of the placenta exhibited features intermediate between those of the giraffe and bovine; *Dolichos biflorus* agglutinin binding – strong in the bovine BNC and absent in the giraffe – was evident in only a subpopulation of BNC while binding to blood vessels, as in the giraffe. Binding of *Phytolacca americana* agglutinin resembled that of the giraffe and okapi whereas many other glycans were found in all four clades. PAG antigens were similar to bovine and okapi but not giraffe. In summary, although the pronghorn outwardly resembles an antelope, placental BNC show giraffid features. Although each clade has its own individual characteristics, there are far more similarities than differences between them, emphasizing the common ancestry of all four clades.

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1. Introduction

The pronghorn (*Antilocapra americana*, Antilocapridae) is an artiodactyl mammal indigenous to North America and is often referred to locally as an antelope. However, phylogenetically Antilocapridae occurs in a superfamily, Giraffoidea, with giraffes and okapi (Giraffidae). In the Pleistocene period, there were 12 taxa of the family Antilocapridae but now the pronghorn is the only extant species. It bears characteristic forked horns [1] that are covered in skin as in giraffes, but in the pronghorn this becomes a keratinous sheath which is shed and regrown on an annual basis [2].

An important feature of the ruminant placenta is the fetal chorionic binucleate cell (BNC) which migrates across the microvillous membrane to fuse with maternal cells, forming fetomaternal trinucleate cells or syncytial masses [3]. These binucleate cells contain heavily glycosylated granules which have been shown to contain placental lactogens which, on migration, pass over to the maternal circulation [4]. Recently, we showed that the placental binucleate cell (BNC) of the giraffe and okapi has a different pattern of glycosylation from other ruminant BNCs that we have studied [5]: greater malayan chevrotain (Tragulidae); fallow deer, red deer, Chinese water deer (Cervidae), domestic goat, springbok, impala, domestic cow and sheep (Bovidae) with little or no expression of terminal αN-acetylgalactosamine bound by *Dolichos biflorus* and *Vicia villosa* agglutinins which instead bind to placental blood vessels [6]. We also demonstrated different patterns of protein expression in the BNC [7].

It appeared that Giraffidae BNC developed different pathways in their glycan biosynthesis and protein expression following their split from the Bovidae, with further differences evolving as okapi and giraffe diverged from each other. Because the pronghorn-

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giraffe clade (or Antilocapridae-Giraffidae clade) diverged from Bovidae [8], it is possible that pronghorn BNC might be different from bovine BNC. We therefore examined placentae from six specimens in order to characterise the glycosylation and protein expression of the binucleate cells and to compare them with those from the giraffe, okapi and bovine.

2. Materials and methods

2.1. Animals

All procedures for collection of animals and tissues were approved by the Fort Keogh Institutional Animal Care and Use Committee (IACUC No. 032415-1). The six pronghorn placental samples (Table 1) were collected and fixed within 20 min after death as part of a wider investigation of pronghorn biology carried out in eastern Montana. Whole placentomes, consisting of fetal cotyledons in close association via microvillous interdigitation with maternal caruncles that form button-like outgrowths on the surface of the uterus, were placed at 4 °C in Surgipath I B F fixative (isopropyl alcohol, methanol, barium chloride and <3% formaldehyde, Leica Biosystems Inc, Buffalo Grove, IL 60089, USA) for 14 days. Central slices of each pronghorn placentome were then cut, put into fresh Surgipath fixative and sent to the UK. The six pronghorn samples had crown rump lengths (from the top of the head to the rump just above the tail) of one of twin fetuses extending from 277 to 318 cm (approximately 160–190 days old, gestation being 240 days). “Matchstick” samples from the central region of each placentome slice from maternal to fetal edge were embedded in epoxy resin.

2.2. Lectin histochemistry

Sections 0.75 μm thick were cut, deresinated in sodium ethoxide, and stained with a panel of 23 biotinylated lectins (see Table 2 for details) at 10 μg/ml as previously described [9] except that SNA-1 was used at a concentration of 50 μg/ml. Some deresinated sections were treated with Type VI neuraminidase (Sigma, from *Clostridium perfringens*) in 0.2 M acetate buffer, pH 5.5, with 1% (w/v) calcium chloride at 37 °C for 2 h [10] to remove terminal sialic acid prior to lectin staining. This revealed glycan residues that were previously masked by the sialic acid. Controls were as previously described [9]. For BSA-II staining for amylase-sensitive glycogen [11], the trypsinisation step was omitted and staining was controlled by digestion in 1% amylase (Sigma, UK) for 20 min at 37 °C following by washing in running water for 10 min. Sections were compared with those of giraffe, okapi and bovine (*Bos taurus*) from our previous study [6] which had been embedded and stained with the same panel of lectins in a similar manner. Subsequently, a series of 8 serial sections was cut from each pronghorn specimen and stained with lectins of particular interest (UEA-1, l-PHA, DBA, VVA, ECA, PAA and WGA) so that specific BNC cells could be tracked to determine glycan characteristics for individual cells. Specific BNC and villi were visualized for the same tissue by overlaying identical fields following staining procedures.

Table 1
Pronghorn twin fetal characteristics.

Specimen number	Crown-Rump Lengths (mm)	Average weight (g)
1	307/301	1134
2	290/290	1025
3	288/290	1080
4	284/290	995
5	283/277	900
6	318/295	1000

2.3. Immunocytochemistry

Samples were postfixed overnight in 4% (para)formaldehyde in PBS with or without 1% glutaraldehyde before epoxy embedding. Semithin sections were cut, picked up on cover glass squares treated with APES and deresinated in sodium ethoxide. The cover glass squares were then floated section side down on drops of antibody followed by immunogold colloid (goat anti-rabbit G5, Jackson Immunoresearch Labs, USA) then intensified with silver reagent (Aurion, Wagenigen, Netherlands). The antibodies used were to Pregnancy Associated Glycoproteins (PAGs) Ovine PAG-1 and Bovine PAG-2 [12] used at a dilution of 1:1000, SBU-3 [13,14], bovine Placental Lactogen (bPL) and prolactin (PRL) [7], all at 1:100. Controls with buffer substituted for antibody showed no significant labelling. The two postfixations produced similar results.

3. Results

3.1. General structure of the placentome

As in all ruminant placentomes, development starts from a flat apposition of trophoblast and uterine epithelium. Mutual growth of the fetal and maternal layers produces placentomes consisting of chorionic villi interdigitating with maternal villi. The fetal digitiform primary villi branch off short, leaf-like secondary villi at right angles to the primary ones while the endometrial villi contain secondary villi to house the secondary fetal villi (Fig. 1A and B). The maternal villi are covered with uterine epithelium underneath which are small blood vessels running along the stroma, while the trophoblast covers primary fetal villi that have a similar subepithelial capillary network with a broader and more loosely arranged mesenchymal core through which run large blood vessels.

The bases of the fetal villi have more regularly arranged columnar cells than cells on the villus sides, similar to those of the arcade regions at the very tips of the villi which are continuous with the flat intercotyledonary area of the placenta (Fig. 1A) on the fetal side, which joins up adjacent cotyledons.

3.2. Lectin histochemistry of binucleate cells (BNC)

Lectin histochemistry of BNC is summarised in Table 2. There was little detectable difference in the results between specimens and two (specimens 1 and 6) are presented in the table as typical examples. There was, however, great variability both in the distribution of BNC stained (Fig. 1C and D) and in their staining intensity with the various lectins (Fig. 1E–H). BNC in the basal regions tended to be smaller than those in the secondary villi themselves while in the arcade region they were large with rather dispersed granules. LEA (Fig. 1C), ECA with neuraminidase, WGA, and PAA both with or without neuraminidase treatment stained the majority of BNC throughout the placentome. In contrast to this, DBA (Fig. 1D) and VVA, did not stain the BNC in in the basal areas or arcade region though staining of cells in secondary villi was strong. This was also evident to some extent with l-PHA, ECA without neuraminidase (Fig. 1E, H) and WFA, where some generally small stained cells were evident in the basal regions but there were more at the tips of the secondary villi. Conversely, the few cells that bound UEA-1 and MAA were mainly found in the basal areas and arcade region rather than in the secondary villi and examination of serial sections revealed that the cells that bound UEA-1 more strongly were invariably those with no DBA or VVA staining; likewise strongly stained DBA/VVA cells were generally negative with UEA-1 (Fig. 1F and G). Comparison of lectin binding of the same six cells with l-

Table 2
Lectin binding to BNC in Two Pronghorn, Giraffe, Okapi and Bovine placentae.

Lectin	Source	Major specificity	Pronghorn 1	Pronghorn 6	Giraffe cot'n	Okapi cot'n	Bovine cot'n
CONA	*Canavalia ensiformis*	Glucose or mannose residues in high	3 (4+)	3 (4+)	3 (4+/−)	3–4	3
PSA	*Pisum sativum*	mannose, small, bi- tri- or tetra-	2	2	3	2	1 (4+/−)
e-PHA	*Phaseolus vulgaris - erythroagglutinin*	antennary, bisected or non bisected	3	3	3	3–4	3–4
l-PHA	*Phaseolus vulgaris - leucoagglutinin*	complex N- linked sequences	2-3 (4+)	2-3 (4+)	4	1	4
LTA	*Lotus tetragonolobus*	L-Fucose terminals linked to galactose	1	1	1–2	1	0
UEA-1	*Ulex europaeus-1*	or N-acetyl glucosamine	1–3	1–2	0	1	0
ALA	*Aleuria aurantia*		4	4	4	3–4	4
MPA	*Maclura pomifera*	Galactose/αN-acetyl galactosamine	3	2–3	2-3 (4+/−)	3–4	1/2++
BSA-1B_4	*Bandeirea simplicifolia*-1B_4		1–2	1–2	3–4	2	0–1
AHA	*Arachis hypogaea*	Terminal βGalactose	1–2	1–2	1-2 (4+/−)	2–3	1
AHA + N	*Arachis hypogaea* after neuraminidase	Subterminal βGalactose	1-2 Golgi 4	2 Golgi 4	2–3	2–3	2–3
ECA	*Erythrina cristagalli*	Terminal N-acetyllactosamine	1-3/4	1-3/4	2 (4+/−)	2	1
ECA + N	*Erythrina cristagalli* after neuraminidase	Subterminal N-acetyllactosamine	4	4	3	4	2–3
DBA	*Dolichos biflorus*	αN-acetylgalactosamine terminals	1–4	1–4	0	0	4
VVA	*Vicia villosa*		1–4	1–4	1 (4+/−)	1	2
HPA	*Helix pomatia*		2–3	2–3	2–3	1–2	1 (3+/−)
WFA	*Wisteria floribunda*		2–4	2-3/4	3	2–3	3–4
SBA	*Glycine max*		1–2	1–2	2–3	2	1 (2+/−)
SBA + N	*Glycine max* after neuraminidase	Subterminal αN-acetylgalactosamine	2	2	3	2	3
BSA-II	*Bandeirea simplicifolia*-II	Terminal α and βNAcetyl glucosamine	1 Golgi 4	1 Golgi 4	1 Golgi 4	1–2	1 Golgi 2-3
DSA	*Datura stramonium*	N-acetyl-glucosamine oligomers and/or	3–4	3–4	4	3–4	4
STA	*Solanum tuberosum*	N-acetyl lactosamine	3–4	3–4	3	3	2–3
LEA	*Lycopersicon esculentum*		3–4	3–4	4	1	1–2
PAA	*Phyttolacca americana*		2-3*	2-3*	3–4	3–4	1 (3+/−)
PAA + N	*Phyttolacca americana* after neuraminidase	Terminal/subterminal N-acetyl-glucosamine oligomers and/or N-acetyl lactosamine.	3*	3*	3–4	3–4	1-2 (3+/−)
WGA	*Triticum vulgaris*	N-acetyllactosamine, di-N-acetyl chitobiose, sialic acid	1-3*	1-3*	3–4	3–4	3–4
WGA + N	*Triticum vulgaris* after neuraminidase	N-acetyllactosamine, di-N-acetyl chitobiose	1-3*	1-3*	3–4	3–4	1-2 (3+/−)
SNA-1	*Sambucus nigra*	Terminal α2,6-linked sialic acid/N-acetyl galactosamine	3–4	3	3–4	3–4	1–2
SNA-1 + N	*Sambucus nigra* after neuraminidase	N-acetyl galactosamine	3–4	3	2	3–4	2
MAA	*Maackia amurensis*	Terminal α2,3-linked sialic acid	2	1-occ3	2–3	1–2	4
MAA + N	*Maackia amurensis* after neuraminidase		0	0	1	1	1–2

Key: 0: negative, 1: weak, 2: moderate, 3: strong, 4: intense lectin binding. Bracket: presence of sparse dark granules in cells. cot'n: cotyledon. *: 4 when merged with maternal epithelium. +N: after neuraminidase.

PHA, UEA-1, DBA and ECA can be seen (Fig. 1E–H).

In contrast to the staining of only small numbers of BNC with DBA and VVA, all the blood vessels were very strongly stained by these lectins, both in maternal and fetal tissues (Fig. 1D), a feature uncommon in many ruminants but present in the giraffe and okapi placenta.

CONA, e-PHA, ALA, DSA, STA and SNA-1 bound to the BNC but they were hard to distinguish from the uninucleate cells which also bound these lectins. PSA, LTA, MPA, HPA, AHA and SBA (both lectins with and without neuraminidase) and MAA bound weakly to moderately and with slight variations that were not very different from the uninuclear cells. After neuraminidase, the background stain was slightly reduced with SNA-1 making the BNC in the secondary villi more prominent.

BNC that had migrated into and fused with the maternal uterine epithelium were particularly well shown by the fixation used. Migrated BNC (actually a BNC fused with a uterine cell, i.e. a trinucleate cell (TNC) or, in some cases, a syncytium containing several nuclei) showed polarisation of granules toward maternal vessels (Fig. 2A) into which, after migration (Fig. 2B) and granule exocytosis, the contents would diffuse. PAA, l-PHA, AHA after neuraminidase, MAA and WGA generally bound very strongly to TNC granules (Fig. 2A, C, D). Fig. 2C depicts a sequence of degranulation in TNC cells stained with MAA which shows the blood vessels particularly well.

One interesting feature was the amylase-resistant binding of BSA-II to selected cisternae (probably the cis face) of BNC Golgi bodies (Fig. 2E); AHA after neuraminidase bound to a much wider proportion of saccules including trans cisternae (Fig. 2F). Other lectins such as UEA-1, DBA and MAA, also showed occasional binding to Golgi cisternae, but this was extremely variable and generally more diffuse.

Comparison of pronghorn BNC lectin staining with the results of giraffe and okapi BNC (see Table 2) showed some similarities and some differences. DBA binding, absent in the giraffe and okapi, was also absent in the majority of the BNC in the pronghorn - only 30–40% of the BNC bound, and these were away from the basal regions of secondary villi.

The strong staining of many BNC with LEA was also a feature of the giraffe, though not okapi BNC, and was only weakly found in the bovine, while PAA bound strongly in pronghorn, giraffe and okapi but not in the bovine. The levels of binding of l-PHA, UEA-1, VVA and ECA were characteristic of the Pronghorn.

The selective staining of Golgi cisternae by BSA-II was also present in giraffe though not okapi, but this could have been due to poor fixation of the okapi tissue. Some staining was evident in the bovine but it was also present elsewhere in some cells and was not restricted to the Golgi of BNC.

3.3. Immunocytochemistry of BNC

Using Pregnancy Associated Glycoprotein antibodies Ovine PAG-1 and Bovine PAG-2, (Fig. 3A–B), the pattern of BNC labelling in the pronghorn was very similar to that which we have previously

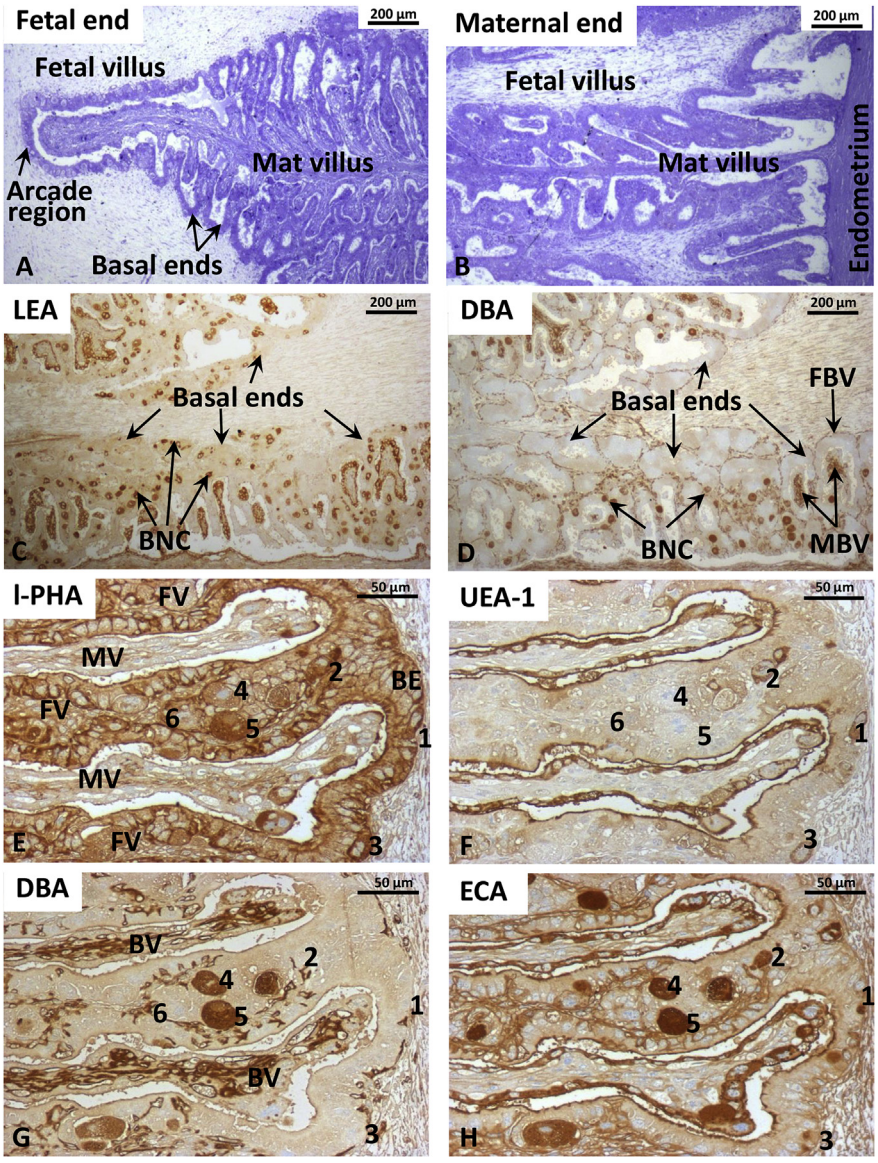

Fig. 1. Toluidine blue and lectin staining to show general structure of pronghorn placentomes. A) The fetal end showing the arcade region and basal ends of secondary villi which interdigitate with those from the maternal villus (Mat villus) running along the centre of the image. The stroma of the maternal villus is always denser than that of the fetal villus. Scale bar: 200 μm. B) The maternal end of the placentome with processes forming villi running between and interdigitating with the thicker fetal villi. Scale bar: 200 μm. C) Section stained with LEA showing numerous binucleate cells (BNC) in the fetal villi, including the curved basal ends. Uninucleate cells do not stain. The maternal epithelial cells strongly bind this lectin. BNC: binucleate cells. Scale bar: 100 μm. D) With DBA, only a subpopulation of BNC bind the lectin, and none are present in the basal ends. Both fetal (FBV) and maternal blood vessels (MBV) also bind DBA. BNC: binucleate cells. Scale bar: 100 μm. E-H) Serial sections stained with l-PHA, UEA-1, DBA and ECA showing numbered cells bind the lectins to different degrees. Those strongly binding DBA are not stained with UEA-1, which tend to be mainly in the basal ends of the villi; l-PHA and ECA tend to bind most, but not all, BNC. MV: maternal villus, FV: fetal villus, BV: blood vessel, BE: basal end of the villus. Scale bars: 50 μm.

demonstrated in Tragulids, Bovids and Cervids [3]. All showed a uniform distribution of positive BNC throughout the placentome, quite unlike the unique regional distribution seen in the Giraffids [7]. In contrast, neither the giraffe nor the pronghorn BNC labelled with SBU-3 (Fig. 3C), a monoclonal antibody which is strongly expressed in the BNC of the other three clades. Prolactin was only found in Giraffid BNC and it was restricted to the fetal end of the placentome, being negative in the pronghorn (Fig. 3D) and okapi. These results are summarised in Table 3.

4. Discussion

The position of the pronghorn in relation to the Cervidae, Bovidae and Giraffidae has been disputed for some time. The pronghorn is Bovid- and Giraffoid-like in that they both have permanent bony horns and not annually grown and shed antlers like all Cervids. In addition Bovids and Giraffids have over fifty placentomes per placenta, whereas Cervids rarely have more than five or six; in pronghorn the number ranges from 46 to 180, with an average of 92 [15]. However, most anatomical characters used to describe the pronghorn and its fossil relatives seem to place them close to the deer family (Cervidae) [16] and it does resemble an antelope in appearance [17]. Recent phylogenetic analysis, however, indicates that the pronghorn family is most closely related to the Giraffidae, forming a sister group to Cervidae and Bovidae, or a sister group to all other horned ruminants [8,16,18,19] rather than being classified in either Bovidae [2] or Cervidae [20] although the placental type and permanent bony horns favour the Bovid

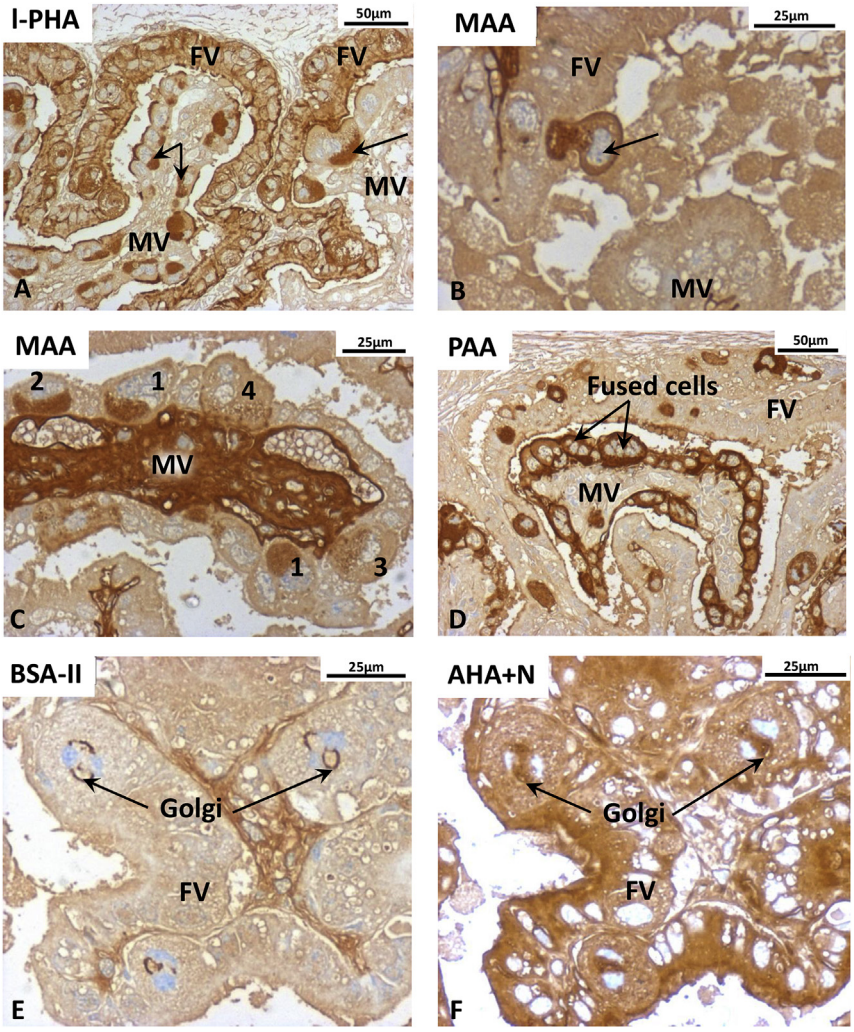

Fig. 2. Features of binucleate cell glycosylation. A) BNC stained with l-PHA that have fused with maternal epithelium (arrows) show polarisation of their granules toward maternal blood vessels in the stroma of the villi. Scale bar: 50 µm. B) A BNC stained with MAA (arrow) in the process of migrating out of the trophoblast layer towards a maternal villus which is poorly preserved in this region. Scale bar: 25 µm. C) Various TNC stained with MAA in different stages of progressive (1–4) degranulation into maternal blood stromal compartment. Scale bar: 25 µm. D) PAA lectin binds to fused tri/multinucleate cells of the maternal uterine epithelium. Scale bar: 50 µm. E) BSA-II binds strongly to selected Golgi cisternae of BNC. Scale bar: 25 µm. F) AHA after neuraminidase binds strongly to a wide area of Golgi cisternae in BNC. Scale bar: 25 µm. MV: maternal villus FV: fetal villus.

classification rather than the Cervids. DNA investigations indicate Antilocapridae is a legitimate family of its own [21] and that its resemblance to the Bovid family of antelopes of Africa and Eurasia is a feature of convergent evolution.

It was thus of interest to examine the placenta and glycosylation of the binucleate cells of the pronghorn as those of the Giraffidae have been shown to have unique characteristics when compared with other ruminants which have a highly conserved pattern of glycosylation [5].

Our glycoprotein analysis has come up with some interesting features, indicating that the pronghorn BNC has some features of the Giraffidae but also some of the Bovidae and other ruminants as well as features of its own. Our detection of H-type 2 antigen (αL-Fuc (1,2)Galβ1,4- GlcNAcβ1-) indicated by UEA-1 binding in sparse cells of the basal areas of secondary villi appears to be unusual in ruminants, and in pronghorn was generally associated with undetectable levels of GalNAcα1,3(LFucα1,2)-Galß1,3/4Glc- NAcβ1-) which is bound by DBA. Terminal α2,3-linked sialic acid-containing cells which bound MAA were also to be occasionally found mainly in the basal and arcade regions, and this was also found strongly in fused cells. The presence of this may be responsible for the increased numbers of ECA-binding BNC observed after neuraminidase staining, especially in basal areas of secondary villi and in the arcade region. Fucosylation was also more prominent here in the BNC. The small size of the cells here, and their staining characteristics that differ from other BNC, suggests that these cells may be immature. It might also be that the absence of cells stained with DBA in the basal region of secondary villi may be due to the fact that this area of trophoblast is similar to the arcade region of intercotyledonary trophoblast, cells of which have a different function to villous trophoblast. However, other BNC glycans appeared to be expressed in both inter- and placentomal areas.

Tri/tetra-antennary, non-bisected N-glycan (l-PHA) was not as strongly expressed as in most other ruminants, a feature found even more so in the okapi where it was almost completely absent, and the strong expression of Galβ1,4GlcNAcβ1- (ECA), with some sub-terminal residues mainly in the basal secondary villi and arcades, was another unique feature. Although non-bisected bi/tri-antennary, complex N- linked sequences (PSA) found in pronghorn, giraffe and okapi were not present in the bovine, they have been described in BNC of other ruminants [5].

The glycans expressed more in the BNC compared to

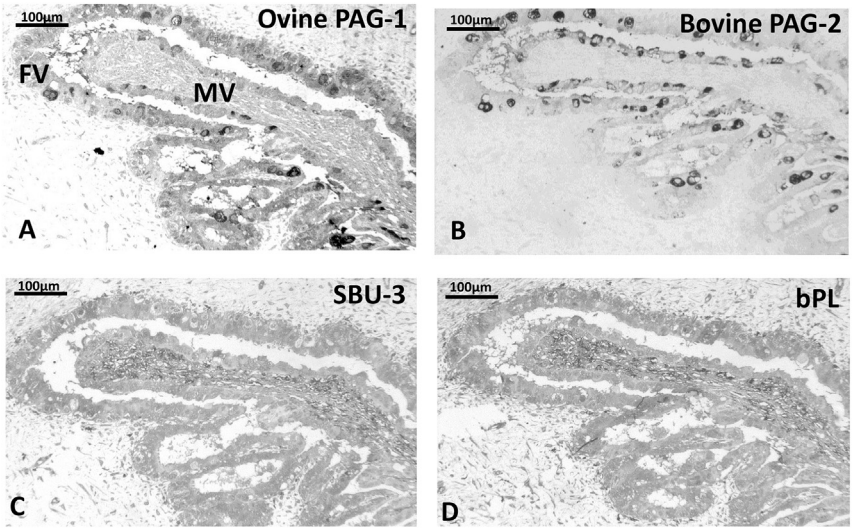

Fig. 3. Immunocytochemistry of BNC in pronghorn. A-B) Pregnancy associated glycoprotein antibodies Ovine PAG-1 and Bovine PAG-2 bind to pronghorn BNC. MV: maternal villus, FV: fetal villus. C) SBU-3 antigen is not expressed in pronghorn BNC. D) Bovine Prolactin antibody does not bind to pronghorn BNC. Scale bars A–D: 100 µm.

Table 3
Immunocytochemistry of BNC.

Antibody	Bovine	Pronghorn	Giraffe	Okapi
Ovine PAG-1	+	+	−	+
Bovine PAG-2	+	+	+[a]	+
bPL	+	−	+[a]	−
SBU-3	+	−	−	−
PRL	−	−	+	−

[a] Fetal end only.

uninucleate trophoblast cells appeared to be mainly N-acetyl glucosamine oligomers as shown by the strong staining with LEA, PAA and WGA, plus terminal and subterminal N-acetyl lactosamine bound by ECA before and after neuraminidase pretreatment. The fixation protocol used clearly showed the polarisation and concentration of BNC granules once they had migrated across to the maternal side and fused with uterine epithelium to form TNC or syncytia. This was most clearly observed with l-PHA but careful examination with ECA, PAA, MAA and WGA staining revealed similar findings. This process ensured that the hormonal contents of the granules would thus pass into the maternal circulation as has been previously demonstrated in the ovine and bovine [4,12,22]. An unusual observation was the precise localisation of binding of BSA-II to selected (cis) cisternae of the Golgi apparatus, suggesting the site of post-translation modification of the secretory proteins with terminal α and β N-acetyl glucosamine [23]. Other lectins bound to a more extensive network of Golgi cisternae, for instance, AHA after neuraminidase.

The immunocytochemical results clearly show that the pronghorn BNCs contain the two PAG antigens which are found in BNCs throughout the placentome and the pattern of labelling is very similar to that previously demonstrated in Tragulids, Bovids and Cervids [3,12]. In contrast, only Bovine PAG2 is found in the giraffe, so in this respect the pronghorn resembles the bovine more than giraffe. Interestingly, the okapi BNC also expresses both PAG antigens but not bPl. There was no evidence for any placentomal regional distribution of BNC content or prolactin antigen presence such as that reported in the Giraffids [7]. However there is good evidence for the close relationship between giraffes and pronghorns in the absence of any binding of the SBU-3 antibody to the BNC granules in either. SBU-3 has been shown to recognise a tri/tetra antennary carbohydrate epitope [14] characteristic of the Bovids and Cervids and its absence indicates significant differences in glycoprotein expression between the clades.

In summary, although the pronghorn outwardly resembles an antelope, and each clade shows individual characteristics, there are far more similarities of the placentomes than differences between them, emphasizing the common ancestry of all four clades. The comparative data described here are, it should be noted, for phenotypes alone. These may reflect ancestry and phylogeny, or may reflect independently evolved characteristics, with giraffe and pronghorn having similar placental characters due to shared common ancestry or each having evolved them separately.

Conflict of interest

We wish to confirm that there are no conflict of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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