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The immunolocalization of Galectin-1 and Progesterone-Induced Blocking Factor (PIBF) in equine trophoblast: Possible roles in trophoblast invasion and the immunological protection of pregnancy

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ABSTRACT

Introduction: The proteins galectin-1 and Progesterone Induced Blocking Factor (PIBF) are present on human and murine trophoblast and are thought to influence both immunomodulation and trophoblast invasion. In equids, the invasive component of the placenta, the endometrial cups, stimulate maternal cell-mediated and humoral immune responses. It was therefore of interest to know if galectin-1 or PIBF could be immunolocalised to the invasive and/or non-invasive components of the equine placenta.
Materials: Horse and mule (♀ horse X ♂ donkey) embryos and placental tissues between Days 12 and 124 of gestation were stained immunohistochemically with antibodies raised against galectin-1 and PIBF. *Results:* Galectin-1 stained the non-invasive trophoblast between Days 15 and 20 but thereafter stained only the invasive trophoblast cells of the chorionic girdle, both before and after they invaded the endometrium to form the endometrial cups. PIBF, on the other hand, stained both the invasive and non-invasive trophoblast throughout the period of gestation studied. Of particular interest was the relative lack of staining of the endometrial cup cells in mule compared to horse pregnancies for galectin-1 and PIBF prior to the earlier and more rapid death and desquamation of the mule cup cells. *Discussion:* The expression of galectin-1 and PIBF proteins in equine trophoblast and the marked difference in lifespan between the endometrial cups in intraspecies horse versus interspecies mule pregnancies support a likely role for these two proteins protecting the fetal trophoblast from maternal immune attack and/or modulation of the invasiveness of endometrial cup cells.

1. Introduction

The mechanisms employed by female equids to overcome the potential risk of the allogeneic conceptus being rejected immunologically by its mother in early pregnancy are particularly interesting for two reasons. First, many of the phenotypically diverse equine species can interbreed to produce viable, although sterile, hybrid offspring [1]. Furthermore, the horse, donkey and zebra can all accept and carry to term extraspecific pregnancies from each other created by embryo transfer [2–4].

Second, union of the fetal allantochorion with the maternal endometrium to commence the process of placentation does not begin until as late as Day 40 after ovulation [5], prior to which the enlarging conceptus remains spherical and unattached within the uterine lumen. At Day 37, a ribbon-like portion of the trophoblast, the chorionic girdle [6,7], assumes an invasive phenotype and the binucleate girdle cells [8] invade the overlying endometrium [9] to form discrete, ulcer-like protuberances known as endometrial cups [10] arranged in a circle at the base of the gravid uterine horn [11]. Invasion of the endometrium by the chorionic girdle and cup cells is transient and shows both spatial and temporal control. The resulting cup cells secrete the gonadotrophic hormone, equine Chorionic Gonadotrophin (eCG) [12] into the maternal circulation to stimulate the development of accessory corpora lutea (CL) [13] which persist until mid-gestation and maintain the pregnancy state until the allantochorionic placenta is sufficiently well established and secreting enough progestagens to assume this role [14].

The horse is unusual in that, during early pregnancy, functional alloantigens are expressed on the invasive chorionic girdle cells and the endometrial cup cells they transform into. They take the form of polymorphic maternal and paternal MHC class I antigens [15–17] and species-specific antigens [18] and their presence results in vigorous humoral [19] and cell-mediated [18,20] maternal immune responses

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mounted against the endometrial cups, the latter being responsible for shortening the secretory lifespan of the cups and hastening their death and dehiscence from the surface of the endometrium between Days 100 and 140 of gestation [12,20]. This process of necrosis and rejection of the endometrial cups occurs more rapidly in mares carrying interspecies mule, than normal intraspecies horse, conceptuses [20].

Galectin-1, an immunoregulatory member of a family of glycan-binding proteins, has been shown to be capable of controlling the proliferation of effector T cells [21] and blocking the secretion of immunomodulatory cytokines [22]. It can also suppress T helper cell (Th) 1 dependant inflammation *in vivo* [23] and it plays a significant role in allowing tumours to escape immune rejection [24]. Galectin-1 is present abundantly in the reproductive tracts of mice [25] and women [26] and Blois et al. [27] demonstrated that pregnancy failure in mice showing reduced expression of galectin-1 expression in the placenta could be prevented by exogenous treatment with galectin-1. Subsequently, the same group showed reduced circulatory levels of galectin-1 in women undergoing early abortion and the expression of galectin-1 on the trophectoderm and inner cell mass of preimplantation human embryos. They proposed that galectin-1 plays an important role in the immune regulation of human pregnancy by modulating the expression of Human Leucocyte Antigen-G (HLA-G) on trophoblast cells [28].

Galectin-1 mediates its effects via binding to the beta-galactosides, including lactose and LacNAc, although other ligands have been reported, including the carbohydrate portion of GM1 and alpha-galactosides [29]. These are present in many cell-surface and extra-cellular matrix (ECM) glycoproteins and since numerous different galectin-1 ligands have been identified at the materno-fetal interface [29], it is perhaps not surprising that galectin-1 plays many roles during pregnancy. For example, in mice in which galectin-1 is synthesised by the trophectoderm of expanded blastocysts prior to implantation, Poirier et al. [30] suggested that it plays a role in attaching the embryo to the uterine epithelium. Similarly, *in vitro* models have demonstrated that galectin-1 can stimulate the adhesion and invasion of trophoblast cells [31]. Furthermore, in women galectin-1 shows differential expression depending on the lineage of the trophoblast suggesting it may have a function in the process of trophoblast differentiation during placentation [32,33]. In first trimester placenta, galectin-1 is localised to the villous cytotrophoblast (CTB) where it plays a role in stimulating syncytium formation [32,34].

Furthermore, villous CTB that proceeds along the invasive pathway shows increased galectin-1 expression [28,35]. Other *in vitro* studies have demonstrated reduced invasiveness by extravillous trophoblast when cultured in the presence of lactose, an inhibitory sugar which prevents the lectin-type interactions of galectins, including galectin-1 [36]. In addition, the invasiveness of tumour cells is influenced by galectin-1 [37,38].

A further immunomodulatory molecule present during pregnancy in women is the 34 kDa protein, Progesterone Induced Blocking Factor (PIBF), secreted by both peripheral blood lymphocytes and trophoblast cells [39,40]. It has been shown to downregulate natural killer (NK) cell activity [41] and influence the T helper cell 1: T helper cell 2 (Th1:Th2) balance by which it contributes to the decreased cell-mediated responsiveness during pregnancy [42]. In women, receptors for PIBF have been shown to be expressed by the majority of lymphocytes [43] and take the form of a heterodimer. This consists of a glycophospatidylinositol (GPI)-anchored PIBF receptor chain and the IL-4R α-chain, which induces Janus kinase 1 (Jak 1) phosphorylation and activation of signal transducers and activator of transcription 6 (STAT6) upon binding of PIBF [44].

Urinary concentrations of PIBF in pregnant women increase continuously from the 7th to the 37th weeks of gestation [45] but they are significantly lower in women with pregnancy toxaemia (pre-eclampsia) [46]. In addition to being measurable in the peripheral circulation PIBF has been localised to the placenta [47].

During first trimester pregnancy in women, the distribution of PIBF staining within the placenta coincides with the sites of trophoblast invasion, the strongest staining occurring in the areas of most intense invasion [47] and its early appearance is accompanied by premature trophoblast invasion [48]. Furthermore, PIBF has been identified in rapidly proliferating cells and tumours [46,49,50]. Curiously, however, although PIBF is expressed highly in normal first-trimester trophoblast, its expression diminishes in hydatidiform moles and choriocarcinomas in proportion to the degree of increased invasiveness of these tumours [51].

In the light of these and other experiments highlighting the role of galectin-1 and PIBF in providing immunological protection for the allogeneic mammalian fetus and potentially influencing trophoblast invasiveness, and in view of the above mentioned endometrial cup reaction demonstrating both humoral and cell-mediated maternal immune responses to foreign antigens expressed on the invasive trophoblast cells of the endometrial cups, it was of interest to examine the expression of galectin-1 and PIBF by both the invasive and non-invasive components of equine trophoblast.

2. Material and methods

2.1. Collection of tissues

With the approval of Sharjah Equine Hospital Ethical Committee, horse embryos and conceptuses were flushed non-surgically from the uteri of pregnant mares between 12 and 34 days after ovulation (Days 12, 15, 18, 20, 24, 34; n ≥ 3 for each stage, n = 15 mares). Pieces of conceptus membranes were dissected free and fixed in 10% v:v formaldehyde solution prior to embedding in paraffin wax. Additional equine placental tissues between Days 12 and 124 of gestation (n = 30 histology blocks), collected and fixed prior to 2007 at the Equine Fertility Unit, Newmarket, UK in accordance with the Animal (Scientific Procedures) Act (1986) were also used.

2.2. Immunohistochemistry

Sections (5 μm) of each block were layered onto positively charged microscope slides for immunocytochemical staining. Briefly, sections were placed in a 56 °C oven overnight to dewax them. Antigen retrieval was undertaken using a bath of high-pH antigen unmasking solution (Dako PT Link; Dako UK Ltd., Ely, Cambs, UK) in which sections were heated to 97 °C for 20 min. After cooling, the slides were rinsed in neutral buffer and transferred to a Dako Autostainer (Dako Plus Autostainer (Dako UK Ltd) where an automated indirect staining method was performed. The optimally diluted primary and secondary antibodies were incubated for 30 min. The secondary antibody, blocking reagents, buffers, substrate, chromagen and nuclear stain were Envision FLEX reagents (Dako UK Ltd) optimised for use in the Autostainer Plus. After staining the slides were removed from the machine, dehydrated, cleared and mounted in DPX.

Two primary antibodies were used; a mouse monoclonal raised against amino acids 1–45 of the N-terminus of human galectin-1 (galectin-1 (C-8); sc-166618; Santa Cruz Biotechnology Inc) at a concentration of 1:225 and a rabbit polyclonal antiserum raised against an amino acid sequence within aa 735–757 of synthetic human PIBF (ab72118, AbCam Ltd, Cambridge, U.K) at a concentration of 1:50.

The human immunogen the galectin-1 antibody had been raised against showed a 97.8% homology with that of horse galectin-1, with only 1 amino-acid difference in the 45 amino-acid sequence. The exact peptide against which the PIBF antibody was raised was proprietary but the manufacturers predicted that the antibody would work with horse tissue. Negative controls were run by replacing each primary antibody with normal, non-immune rabbit or mouse serum.

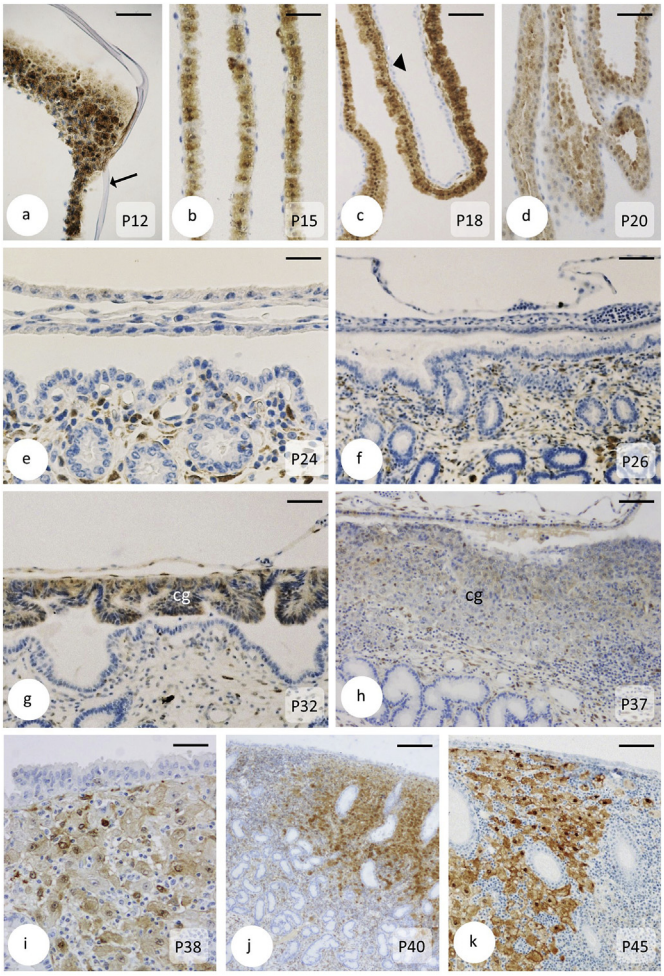

Fig. 1. a–c) Strong staining of the equine trophectoderm by the galectin-I antiserum between 12 and 18 days of gestation. Note the lack of staining of the acellular embryonic capsule (arrow) present in the Day 12 specimen and the hypoblast (arrow head) seen clearly at Day 18 (scale bars; a = 35 μm; b & c = 40 μm). d) Subjectively, a notable decline in staining intensity was observed in Day 20 trophoblast (scale bar = 40 μm). e & f) Positive staining was seen in some cells in the endometrial stroma by the galectin-1 antiserum but a complete absence of staining in the overlying trophoblast of the chorion at 24 and 26 days of gestation (scale bars; e = 40 μm and f = 180 μm). g & h) The chorionic girdle (cg) cells showed pale staining with the galectin-1 antiserum, both at 32 days before invasion of the endometrium (scale bar = 150 μm) and at 37 days when the chorionic girdle (cg) has just attached itself to the endometrium (scale bar = 180 μm). i) The invading girdle cells are now staining more strongly for galectin-1 at Day 38 (scale bar = 40 μm). j & k) The now completely differentiated endometrial cup cells stain very strongly for galectin-1 at 40 and 45 days of gestation (scale bars; j = 200 μm and k = 150 μm).

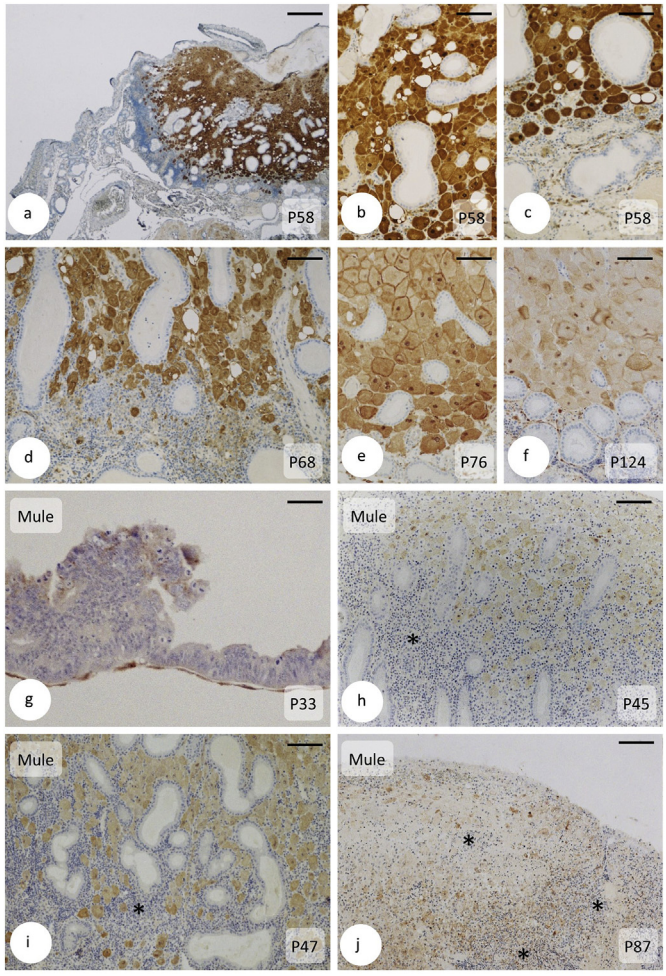

Fig. 2. a) Low power section at one edge of an endometrial cup at 58 days of gestation showing strong staining for galectin-1 in just the large, fetal endometrial cup cells (scale bar = 300 μm). b & c) Sections in the centre (b) and at the base (c) of the 58-day endometrial cup showing strong and precise staining of the fetal endometrial cup cells for galectin-1. The endometrial gland epithelium remains unstained (scale bars = 40 μm). d – f) Sections showing definite weakening of staining intensity in the endometrial cup cells with advancing gestation (scale bars; d = 100 μm, e & f = 40 μm). g) In comparison to the horse, much weaker galectin-1 staining of g) mule (donkey ♂ x horse ♀) chorionic girdle at Day 33 (scale bar = 80 μm) and, h - j) mule endometrial cup cells at 45 (h), 47 (i) and 87 (j) days of gestation (scale bars; h = 180 μm, i = 100 μm, j = 200 μm). Note the much denser accumulations of leucocytes (asterisks) surrounding and invading into the mule cup tissue at these 3 stages.

3. Results

3.1. Immunohistochemical localisation of Galectin-1 and PIBF

3.1.1. Galectin 1

The trophectoderm of embryonic membranes recovered between 12 and 20 days after ovulation stained for galectin-1 (Fig. 1a–d). Subjectively, staining appeared to diminish in intensity at Day 20. Between 24 and 30 days of pregnancy staining was no longer apparent in the non-invasive trophoblast, although individual cells within the endometrial stroma, thought to be macrophages, stained positively (Fig. 1e and f) and continued to do so throughout the period studied. However, pale staining was seen in trophoblast cells of the developing pre-invasion chorionic girdle (Fig. 1g) and weak staining was still evident in the girdle when it had attached to the endometrium around Day 37 (Fig. 1h). As the girdle cells enlarged and began to actively invade the endometrium to form the definitive endometrial cup cells the intensity of staining for galectin-1 increased (Fig. 1i–k). And as the endometrial cup reached full maturity and function, the transformed binucleate fetal endometrial cup cells, but not the non-invasive trophoblast of the allantochorion, stained precisely and strongly for galectin-1 (Fig. 2a–c). Both the nucleus and cytoplasm stained although the peripheral cytoplasm and cell membrane was frequently stained more intensively. Coincidentally with the decline of eCG concentrations in maternal blood and the commencing degeneration of the cup cells due to a combination of the deficiency in blood supply and increasing attack from maternal leukocytes, the intensity of staining of the persisting cup cells steadily decreased. This was most evident in the cytoplasm of the cup cells, in which the nucleus and the cell membrane remained stained for longer (Fig. 2d–f). The eCG-rich cell debris and exocrine endometrial gland secretion accumulated on the surface of the

cups (endometrial cup secretion) did not stain for galectin-1 (Fig. 2a) throughout the cup's lifespan. Likewise, after Day 20 of gestation, the non-invasive trophoblast of the allantochorion remained unstained for galectin-1 in all the samples examined (Fig. 1d–e; 2a).

Subjectively there appeared to be little difference in the staining intensity of the mule (donkey ♂ X horse♀) chorionic girdle (Fig. 2e) compared to that of the horse (Fig. 1f). However, the endometrial cup cells in hybrid mule pregnancies stained less strongly for galectin-1 than their counterparts in normal intraspecies horse pregnancy at similar stages of gestation (Fig. 2f–h) and this was accompanied by a much stronger and more invasive maternal leucocyte reaction against the fetal cup cells. As a result of the appreciably narrower and generally smaller chorionic girdle that develops on the interspecies mule as compared to that of the intraspecies horse, the endometrial cup cells in the mule pregnancies were fewer in number and the cup cells were less tightly packed together in the endometrial stroma than in the horse endometrial cups. In addition, the leucocytes migrated more rapidly into the body of the mule endometrial cups and killed the fetal cup cells so that, by as early as Days 70–80 of gestation, the mule cups were already necrotic and in the process of being sloughed off the surface of the endometrium (Fig. 2h).

Staining was abolished completely when the galectin-1 antibody was replaced by normal, non-immune mouse serum.

3.1.2. Progesterone Induced Blocking Factor (PIBF)

The trophectoderm of the embryonic membranes stained strongly for PIBF activity from Day 12 of gestation onwards (Fig. 3a). However, in contrast to galectin-1, PIBF staining persisted in the non-invasive trophoblast (Fig. 3b) prior to its commencing interdigitation with the endometrium from around Day 40. The invasive chorionic girdle cells also stained with the PIBF antibody and, subjectively, the staining appeared to intensify at the tips of the girdle villi as it matured prior to invasion of the endometrium (Fig. 3c–d). In addition, the epithelial cells lining the lumenal surface of the endometrium and the endometrial glands also stained for PIBF (Fig. 3c) in all sections where maternal tissue was present (i.e. 20 days onwards). After invasion of the chorionic girdle the transformed endometrial cup cells, and leukocytes in the stroma around the edge of the cup, stained for PIBF (Figures e–f). Initially, the regenerating epithelial layer overlying the cup did not stain for PIBF, but it soon began to do so (Fig. 3e–g). Staining within the cup cells and within the epithelium lining the dilated endometrial glands, both within and beneath the cup, was granular in appearance (Fig. 3g–I; Fig. 4a) and it persisted throughout the lifespan of the cups. Staining of the non-invasive trophoblast was less granular and it persisted throughout the study period, as did staining of the lumenal and glandular epithelia (Fig. 3b).

Mule chorionic girdle tissue showed pale staining for PIBF but this was never as intense as the staining of horse chorionic girdle tissue (Fig. 3c) and once the mule girdle had invaded the horse endometrium staining for PIBF was weak to non-existent in the resulting endometrial cup cells (Fig. 3d–f). A sub-population of the leukocytes around and within the cup were stained positively for PIBF at all stages examined (Fig. 3e–f).

Staining was abolished completely when the PIBF antibody was replaced by normal, non-immune rabbit serum (Fig. 4g).

4. Discussion

This paper highlights the presence of two immunomodulatory proteins, galectin-1 and Progesterone Induced Blocking Factor (PIBF), on equine fetal membranes and the invasive endometrial cup cells during early pregnancy.

Galectin-1 showed pronounced biphasic gestation-dependent labelling of trophoblast cells, with intense staining of early non-invasive trophectoderm during Days 12–20 of gestation and staining of invasive trophoblast from Day 30 onwards which continued throughout the 60–80 day lifespan of the endometrial cups.

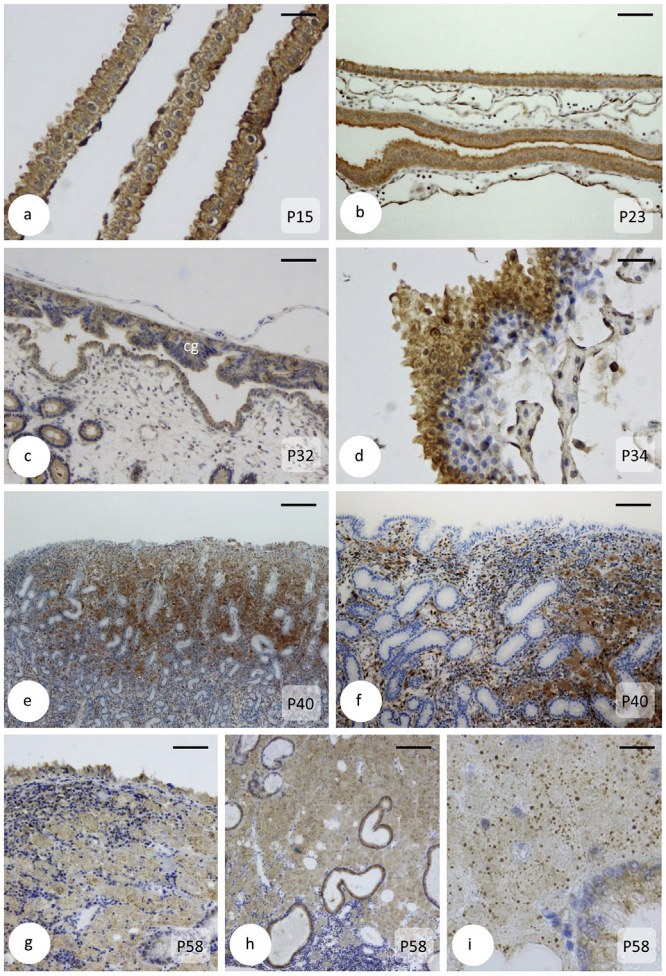

Fig. 3. a & b) Strong positive staining of the trophoblast tissue of horse fetal membranes with the PIBF antiserum at 15 and 23 days of gestation (scale bars; a = 30 μm, b = 50 μm) c & d) The staining for PIBF persists in the chorionic girdle at 32 and 34 days (obliquely cut) of gestation (scale bars; c = 180 μm, d = 30 μm). In contrast to the situation with galectin-1, now the lumenal and glandular epithelia of the endometrium also stain positively for PIBF. e & f) Low and high power sections of an endometrial cup at Day 40 of gestation showing strong positive staining of the fetal cup cells as well as cells within the endometrial stroma (scale bars; e = 200 μm, f = 180 μm). g-i) Low, medium and high power sections of a Day 58 endometrial cup stained with the PIBF antiserum. The gland epithelium show positive cytoplasmic staining. Within the cup cells the positive staining for PIBF is granular in appearance (scale bars; g = 150, h = 100 μm, i = 30 μm).

Given the numerous roles galectin-1 plays during embryo development and pregnancy in women and mice (*reviewed in* Ref. [29]), it is difficult to state conclusively what its exact function in equine pregnancy may be. However, the fact that galectin-1 has been shown to make a pivotal contribution to fetomaternal tolerance in other species by maintaining the balance between pro-inflammatory Th1 cytokines and tolerogenic Th2 cytokines [27,52–54], it seems reasonable to assume it may do likewise in the horse, especially given that galectin-1 is most strongly expressed in the invasive component of the trophoblast.

Chorionic girdle and early endometrial cup cells express polymorphic maternal and paternal MHC class I antigens for a limited period in early equine pregnancy [16,17] against which the mare mounts a strong humoral antibody responses [55], with titres rising higher and earlier in subsequent pregnancies, although only after the chorionic girdle has invaded the endometrium to form the endometrial cups [55,56]. This presence of paternal MHC class I antigens on the

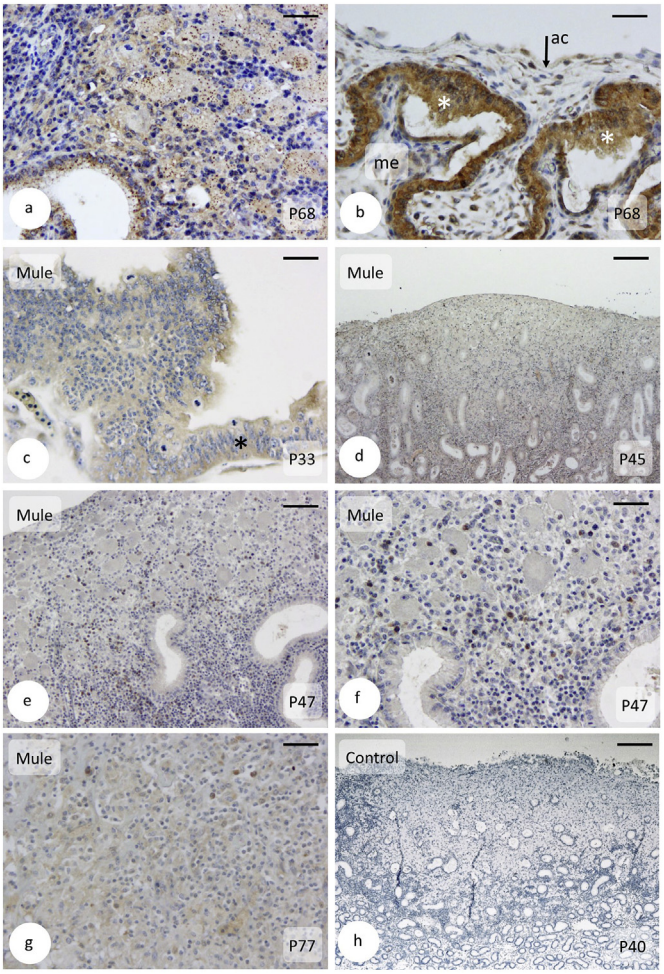

Fig. 4. a & b) High power sections at Day 68 of gestation of, a) horse endometrial cup cells showing the granular PIBF staining pattern and, b) strong staining of the non-invasive trophoblast of the allantochorion (ac) overlying the maternal endometrium (me). Note the two histotroph exchange areolae (asterisks; scale bars = 40 μm). c) Section of a mule 33-day chorionic girdle showing pale staining of the girdle cells and adjacent non-invasive trophoblast (asterisk) with the PIBF antiserum (scale bar = 60 μm). d) Low power section of a 45 day mule endometrial cup showing very pale staining of the fetal cup cells by the PIBF antiserum (scale bar = 200 μm). e & f) Low and higher power sections of a 47 day mule endometrial cup. Note the absence of staining of the fetal cup cells with the PIBF antiserum and the dense accumulation of maternal leucocytes already deeply invaded into the cup tissue as small proportion of which stain with the PIBF antiserum (scale bars; e = 100, f = 45 μm). g) Section of a mule endometrial cup at 77 days of gestation stained with the PIBF antiserum. All the cup cells are now dead and the tissue is heavily invaded by leukocytes, a small proportion of which stained positively (scale bar = 50 μm). h) A control section of a horse endometrial cup at day 40 of gestation (scale bar = 200 μm).

chorionic girdle cells and early cup cells results in large numbers of maternal $CD4^-$ and CD8-positive lymphocytes accumulating within and around the cup, although this cellular immune response does not result in their immediate destruction [57]. No such collection of lymphocytes occurs in the endometrium interdigitating with the non-invasive allantochorion [57] and it is therefore tempting to speculate that the expression of galectin-1 by the cup cells plays a significant, if not pivotal, role in keeping the maternal cell mediated attack at bay during the early life of the endometrial cups. Indeed, the relative lack of expression of galectin-1 by the mule versus horse endometrial cup cells and the much faster destruction of the former by the maternal cell mediated response mounted against them would support such an hypothesis. However, prior to the demise of the endometrial cups, there has been a downregulation of the MHC I antigens on the cup cells and a decline in their eCG secreting capacity, both of which could also play roles in cup death and immune cell regulation.

It has been suggested that galectin-1 is essential to maintain the balance between pro-inflammatory Th1 cytokines and tolerogenic Th2 cytokines during pregnancy in mice and women [27,52–54]. In the mare, however, lymphocytes recovered from the endometrial cups versus those in the peripheral circulation show a marked increase in interferon (IFN)-γ (a Th1 cytokine)-positive lymphocytes within both the total population and the CD8 positive subpopulation, and a decrease in the proportion of lymphocytes expressing interleukin (IL)-4 (a Th2 cytokine) [58]. This is in contrast to the traditional view that pregnancy is associated with a decrease in the ratio of Th1:Th2 cytokines. Hence, if galectin-1 is acting locally to modulate the maternal immune response to the fetal endometrial cup cells, it does not appear to be doing so by the traditional dogma of altering the Th1:Th2 cytokine ratio. However, it has also been proposed that $CD4^+CD25^+$ regulatory T-cells have an essential role in the generation of fetomaternal tolerance in mice [59,60] and humans [61,62]. In galectin-1 deficient mice, which show higher rates of fetal loss than their wild type equivalents, treatment with recombinant galectin-1 prevents such fetal loss and results in the induction of tolerogenic dendritic cells that promote the expansion of IL-10 secreting regulatory T cells (Tregs) [27]. De Mestre et al. [58] found evidence of an increase in $CD4^+$, forkhead box transcription factor (FOXP3)-positive lymphocytes surrounding the endometrial cups compared to those in the peripheral circulation, which they assumed were most likely Treg cells. Hence, it would seem more likely that galectin-1 expression by the endometrial cup cells may act in a similar manner to the aforementioned mouse model to regulate immunotolerance.

In an *in vitro* model, galectin-1 has been shown to have the ability to stimulate the adhesion and invasion of trophoblast cells isolated from first trimester human placentas [63]. Furthermore, the extravillous trophoblast that invades the decidua displays the highest galectin-1 expression [64,65]. In the present study the immunolocalization of galectin-1 to just the discrete invasive portion of the equine placenta may also signify that it modulates trophoblast invasiveness.

The expression of galectin-1 by non-invasive trophoblast before Day 20 of gestation may also play a role in modulating the maternal immune system to prevent it mounting a destructive response against the early equine embryo prior to it becoming stationary at the base of one uterine horn at around Day 16–17 [66]. In the present study the youngest embryo stained for galectin-1 was at Day 12 after ovulation. However, since Swegen et al. [67] showed the presence of galectin-1 in the medium recovered from a 48 h culture of a Day 8 equine embryo, it is clear it is being secreted into the uterine lumen by the embryo prior to Day 12. Furthermore, its detection in the culture medium indicates it can pass through the acellular mucin-like glycoprotein capsule that completely envelops the equine embryo between Days 6.5 and 23 of gestation [68,69]. One theory among several proposed is that the presence of the capsule around the young embryo protects it from maternal immunological recognition and attack [68]. However, this would seem less likely if galectin-1 is acting to modulate any potential maternal immune response.

The absence of galectin-1 protein in the non-invasive trophoblast beyond Day 20 is more difficult to explain if the assumption is made that galectin-1 is secreted to modulate any maternal immune responses at a local level, especially as the reduction in galectin-1 protein expression occurs around the time of disintegration of the capsule to allow a more intimate contact between the trophoblast and the maternal endometrium.

Significantly, however, unlike the chorionic girdle and early endometrial cup cells, the non-invasive trophoblast does not express MHC Class I or II antigens [15,16,70] during this early period of pregnancy.

In women, the differential expression of galectin-1 in different

trophoblast cell lineages make it a candidate for functioning as a stimulator of cell fusion and syncytium formation [28,32], and as a potential supporter of cell-cell interactions needed for exchange processes within the developing trophoblasts [29]. Angiogenesis has also been hypothesised as another process essential in embryonic development which may be influenced by galectin-1 [29]. Which, if any, of these suggested additional functions of galectin-1 in human trophoblast can be extrapolated to the horse remains to be determined.

In contrast to galectin-1, the other immunomodulatory protein studied, PIBF, was immunolocalised to equine trophoblast at all stages of pregnancy examined (Day 12–124). Human trophoblast cells also express PIBF which has been proposed to act both systemically and locally on the maternal immune system during pregnancy [47]. PIBF was described initially as being secreted from progesterone-activated lymphocytes and decidual CD56 $^{+}$ cells in pregnant women, resulting in the downregulation of natural killer (NK) cells and a shift in the Th1:Th2 balance to give dominance of humoral over cellular immunity during pregnancy [39,40,42]. However, as mentioned previously, this change in the Th1: Th2 ratio has not been shown to occur in the mare, either at the placental interface or peripherally [58]. Notwithstanding, a subset of cells within the endometrium adjacent to the young endometrial cup did stain positively with the PIBF antibody and the possibility therefore exists that these may have been leukocytes secreting this protein to modulate the local immune environment.

Like galectin-1, the strong expression of PIBF protein by the horse endometrial cup cells, and a by subpopulation of immune cells around the periphery of the cup, may be related to modulation of the immune response. And as with galectin-1, the decreased expression of PIBF in the cup cells as they aged and eventually died, suggests that PIBF may be related to the cells' ability to fend off untimely attack by the maternal immune system, especially given that the mule endometrial cups showed little to no expression of PIBF and, characteristically, have a much greater cellular immune response mounted against them [18].

PIBF has also been identified in rapidly proliferating cells and tumours [46,49,50] which match the phenotypes of chorionic girdle and endometrial cup cells. Indeed, the distribution of PIBF within human first trimester decidua coincides with the sites of trophoblast invasion, showing the strongest PIBF expression in the extravillous trophoblast [47]. Studies examining the possible mode of action of PIBF in tumour invasion concluded that PIBF facilitates invasion of the tumour cells by activating the genes of molecules, such as epidermal growth factor (EGF), that initiate invasion signalling. Secreted proteins then bind to their receptors to activate signalling pathways which could trigger the expression of invasion promoting molecules such as the metalloproteinases (MMP)-9 and -2 [71]. In the horse, both EGF and its receptor are expressed on the invading chorionic girdle cells and on the resulting cup cells [72] and metalloproteinase activity has been demonstrated on chorionic girdle cells [73]. Hence, PIBF may be working to assist in the invasion of the endometrium by the chorionic girdle cells in a manner similar to that described by Halasz et al. [71].

The granular staining pattern shown by PIBF within the cytoplasm of the mature cup cells was particularly striking. PIBF positive cytoplasmic granules have been noted previously in human pre-decidual multipotent stromal cells, with the suggestion that the specific granular pattern of positive staining indicated secretion of PIBF by the cells [74]. The cytoplasm of the mature endometrial cup cell contains many mitochondria, rough endoplasmic reticulum cisternae and a large Golgi apparatus [8]. Equine CG is is localised in the Golgi cisternae, in small dense granules, with the suggestion that its release would be an exocytotic mechanism in common with other protein hormones [8]. It is not possible to say if the PIBF positive granules are co-localised with eCG positive ones in the cup cells. However, it is likely that secretion of PIBF is via the same exocytotic mechanism.

In contrast, the non-invasive trophoblast showed non-granular staining of the cytoplasm with the PIBF antibody. Its continued presence on the non-invasive trophoblast throughout the period studied suggests that it either has a long-term role during pregnancy in modulating the immune response and/or plays a role in the materno-feto relationship. It is known that trophoblast cells in the human placenta can express PIBF proteins of varying kDa during the first trimester [47]. In mice full-length PIBF shows a peri-nuclear localisation and is over-expressed in rapidly proliferating cells [49] and has been reported to regulate trophoblast and tumour cells invasiveness [51,71,75]. Whereas, smaller isoforms are localised to the cytoplasm and are believed to account for the immunological effects of PIBF [76] and the modulation of other aspects of the materno-feto relationship [44,77]. In the mare, we do not currently know if multiple PIBF isoforms exist with different roles in pregnancy, but the varying locations of PIBF within both the non-invasive and invasive trophoblast cells during early pregnancy would hint at this possibility.

Although the mule cup is considerably smaller than that of the horse, due to the considerably narrower chorionic girdle which provides the invasive cells that form the cup [18], the lack of PIBF positive cup cells was a very noticeable feature. Perhaps this, in part, explains why it is so readily destroyed by the lymphocytes and has a shorter lifespan than the horse cup. If both galectin-1 and PIBF are playing an immunomodulatory or invasive role in the endometrial cup the reduction in staining in the mule cup versus the horse fits well with this hypothesis.

Both galectin-1 and PIBF have been studied predominantly in human and mouse pregnancy, and little published information exists as to their presence in other mammals. Both have been demonstrated in the endometrium of cattle in early pregnancy [78,79] but not on the placental membranes. The present paper describes the expression of both galectin-1 and PIBF in the trophoblast of a species other than the human and mouse and, given that the equine trophoblast uniquely has both invasive and non-invasive components, it potentially makes an interesting model for further study.

In conclusion, this study showed that both galectin-1 and PIBF are present in equine trophoblast during the first third of gestation. Galectin-1 showed a biphasic expression, being present in the early, non-invasive trophoblast until around Day 20 and then in the invasive trophoblast of the chorionic girdle and the resulting endometrial cup cells from around Day 32. PIBF, on the other hand, was present in both invasive and non-invasive trophoblast throughout the stages of pregnancy examined. The precise role(s) of these two immunomodulatory proteins in altering the immune responses of the pregnant mare to ensure immunotolerance of the paternal antigens of the fetus and governing other developmental processes during early placentation remains to be determined.

Conflicts of interest

None.

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