Markers of equine placental differentiation: insights from gene expression studies

Shavahn Loux¹, Morgane Robles^{2,3,4}, Pascale Chavatte-Palmer^{3,4} and Amanda de Mestre⁵

¹Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, Kentucky, USA, ²INRS Centre Armand-Frappier et Santé Biotechnologique, Laval, Québec, Canada, ³Université Paris-Saclay, UVSQ, INRAE, BREED, Jouy-en-Josas, France, ⁴Ecole Nationale Vétérinaire d'Alfort, BREED, Maisons-Alfort, France and ⁵Comparative Biomedical Sciences, Royal Veterinary College, Hatfield, UK

Correspondence should be addressed to S Loux or A de Mestre; Email: shavahn.loux@uky.edu or ademestre@rvc.ac.uk

Abstract

Development and the subsequent function of the fetal membranes of the equine placenta require both complex and precise regulation of gene expression. Advancements in recent years in bioinformatic techniques have allowed more extensive analyses into gene expression than ever before. This review starts by combining publically available transcriptomic data sets obtained from a range of embryonic, placental and maternal tissues, with previous knowledge of equine placental development and physiology, to gain insights into key gene families relevant to placentation in the horse. Covering the whole of pregnancy, the review covers trophoctoderm, yolk sac, chorionic girdle cells, allantoamnion and allantochorion. In particular, 182 predicted 'early high impact' genes were identified (>100 transcripts per million (TPM) and >100 fold-change) that distinguish between progenitor trophoctoderm, chorionic girdle tissue and allantochorion. Furthermore, 71 genes were identified as enriched in placental tissues (placental TPM > 10, with minimal expression in 12 non-placental TPM < 1), including excellent candidates for functional studies such as IGF1, apolipoproteins, VGLL1, GCM1, CDX2 and FABP4. It is pertinent that future studies should focus on single-cell transcriptomic approaches in order to determine how these changes in gene expression relate to tissue composition and start to better define trophoblast subpopulations in the equine placenta. Future functional characterisation of these genes and pathways will also be key not only to understanding normal placental development and fetal health but also their potential role in pathologies of pregnancy.

Reproduction (2022) **163** R39–R54

Introduction

Equine pregnancy is characterised, among other physiological aspects, by a long gestation (11 months), a late implantation (>35 days) and four distinct placental tissue types. Two short-lived tissues, yolk sac and endometrial cups derived from an invasive chorionic girdle, support the pregnancy for the first 50 and 100 days respectively. The definitive, diffuse epitheliochorial allantochorial placenta in the mare is however the primary tissue that supports a single offspring throughout gestation assisted by the fourth tissue type, the allantoamnion that immediately surrounds the fetus. Placental formation is both complex and unique but, in common with other mammalian species, requires contributions from three cell lineages, ectoderm, endoderm and mesoderm, as described in Fig. 1.

Until 10 years ago, studies of the key molecules involved in regulating equine placental development were limited to targeted candidate-gene PCR and immunohistochemical descriptions of the bilaminar and trilaminar yolk sac, the chorionic girdle and

chorioallantois between 2 months and term. Since the sequencing of the equine genome (Wade *et al.* 2009), we have observed a rapid expansion in the availability of molecular tools and bioinformatic resources that could be now applied to equine studies, allowing an improved understanding of equine placental gene expression and associated regulatory pathways. Similar to other species, 3-dimensional structure, ligand availability, transcription factor and epigenetic modifications are all examples of factors demonstrated to regulate placental gene and/or protein expression in the mare. Identification of these factors that regulate early placental growth, along with their downstream mediators, is crucial not only for our knowledge of what is required for development of a healthy offspring but to also shed light on underlying causes of pregnancy failure and mechanisms of gestational programming of the offspring (Robles *et al.* 2022).

Studies using microarrays and next generation sequencing (NGS) to investigate gene expression in equine placental tissue extend from descriptions of the primary derivative membrane, the blastocyst-derived

Figure 1 Schematic illustrating the four main placental membranes in the mare, their lifespan and how the three cell lineages, ectoderm, endoderm and mesoderm, each contribute to the membranes starting from a day 6 early blastocyst comprised of an inner cell mass (ICM) and trophectoderm (TE). The bilaminar membrane of the yolk sac forms from fusion of the ectoderm and endoderm which is later lined by vascular mesoderm to give rise to the trilaminar yolk sac wall. The chorion forms when the TE is lined by avascular mesoderm around day 14 of pregnancy. The chorion then contributes to the three other placental tissues: the endometrial cups, the allantochorion and allantoamnion. The chorionic girdle trophoblast is derived from a specialised region of the chorion abutting the newly developing allantochorion. The allantochorion forms when the chorion fuses with the allantois composed of endoderm and vascular mesoderm. The chorion folds around the embryo proper to give rise to the amnion which is similarly lined by allantois.

trophectoderm (TE; [Iqbal et al. 2014](#)), to chorionic girdle ([Brosnahan et al. 2012](#), [Read et al. 2018a](#)) and term chorioallantois ([Robles et al. 2018](#), [Loux et al. 2019a](#)). The majority of these studies have focused on characterising tissues of mixed cellular composition emphasising the importance of interpreting these observations alongside our understanding of tissue composition ([Fig. 1](#)) and cell types present ([Fig. 2](#)) in the four main placental tissues in equids.

This review performed a meta-analysis using publically available published transcriptomes to enable us to compare gene expression across multiple placental tissues derived from blastocysts through to term pregnancies with as little bias as possible. We then reviewed this data alongside the published literature and our knowledge of tissue composition to define how expression changes over time within the distinct extra-

embryonic membranes. We then went on to identify genes highly enriched in placental tissue as well as define genes that differentiate the two main lineages of early equine placental tissue, the chorionic girdle and allantochorion.

Materials and methods

In order to compare data for this review from multiple studies whilst minimising bias, raw data were pulled from the Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>), with individual files identified by first searching for *Equus caballus* using RNA as a filter (1550 samples on 11/1/2019). All reproductive tissues derived from healthy individuals were selected, then screened for duplication and quality. When duplicate studies with equivalent tissue types were present (e.g. diestrus endometrium), the study with higher read quality

Figure 2 Day 34 equine conceptus highlighting the location of the chorionic girdle (invasive placenta, upper left), yolk sac (upper right) and allantochorion (definitive placenta, lower right). Hematoxylin and eosin-stained histological sections demonstrate the cellular composition of each membrane at day 34, including the layers that contribute to each tissue. The chorionic girdle cells invade into the endometrium around day 36–38 to form the endometrial cups (lower left histological image) with an arrow highlighting endometrial glands, a circle demonstrating a binucleate cell and an arrowhead showing a cluster of lymphocytes. Scale bar = 100 µm.

and higher read counts was selected. All non-reproductive tissues were selected from the functional annotation of animal genomes project as all tissues sequenced were derived from two young, healthy individuals; tissues selected included major internal organs as well as those with a peripheral reproductive function, including lung, liver, kidney, cerebellum, adrenal gland, skin and adipose. A full list of tissues analysed is described in Table 1, with a total of 78 samples representing 20 different tissues/conditions being ultimately analysed.

Sequencing quality from FASTQ files was assessed using FASTQC, with bases with a Phred score <20 were trimmed using Trim Galore. Trimmed FASTQ files were mapped to EquCab3.0 (Kalbfleisch *et al.* 2018) using STAR-2.5.2b, then annotated with Cufflinks-2.2.1 using the Ensembl v 95 *Equus caballus* annotation file. All programs were run using the default settings unless otherwise specified. To better control for inter-study variability, quantification was performed via transcripts per million (TPM).

As there is necessarily a great deal of variability in sequencing techniques and library preparation between samples, we chose not to rely strictly on differentially expressed genes for any part of our analyses, instead use much more stringent conservative criteria to identify genes of importance. To be defined as high-impact genes, both high expression (>100 TPM) and large differences across tissues (>100 fold-change) were required. To identify placental-enriched genes, moderate to high placental expression (>10 TPM) combined with low expression in all other studied tissues (<1.5 TPM) was the criteria.

Gene expression in placental lineage formation and placental membranes throughout gestation

Trophectoderm

The equine embryo is retained in the oviduct between 5.5 and 6.5 days, entering the uterus as a morula or early

blastocyst. The first report of TE gene expression using an array or NGS technology is at day 8, coinciding with at least 36-h exposure to the endometrial environment. Equine embryonic stem cells are totipotent cells whose initial differentiation results in the formation of a placental lineage (TE) and embryonic lineage (inner cell mass; ICM), with the TE ultimately contributing to all four types of placental membrane in the horse (Fig. 1). Therefore, not surprisingly, gene expression in day 8 blastocysts between TE and ICM is overlapping, with the TE expressing 705 unique transcripts and an additional 10,364 transcripts in common with the ICM (Iqbal *et al.* 2014). The TE is defined by enriched expression of transcription factors such as *CDX2*, *GATA2*, *GATA3*, *TEAD4*, *ELF3* and *TFAP2A* (Iqbal *et al.* 2014), many of which are also genetic signatures in mouse, human and bovine TE (Posfai *et al.* 2019). Similar to early TE of human embryos, day 8 equine TE also express the pluripotency markers, *POU5F1* (Choi *et al.* 2009) and *CDX2*, a pattern that also distinguishes them from mouse embryos which exclusively express these genes in the ICM or TE respectively (Hori *et al.* 2016). Meta-analysis demonstrated that many key genes used to define ICM, TE and primitive endoderm (Iqbal *et al.* 2014) are also expressed in a broad range of tissues (Table 2).

The most highly expressed transcription factor in equine TE is *GATA3* (Iqbal *et al.* 2014), with the meta-analysis indicating that expression is sustained throughout equine placental development (Table 2) consistent with high levels of expression observed in human placenta (Sabon *et al.* 2014). Whilst *in vitro* generated cell lines such as induced TE derived from equine umbilical cord matrix mesenchymal stem cells show a similar gene profile to *ex vivo* TE, they importantly lack expression of *GATA3* (Reinholt *et al.* 2017). It is important to note

Table 1 *Equus caballus*-derived sequencing data used in meta-analysis.

Sample	Replicates	Library Prep	SRA identifier	Reference
Adipose	2	PolyA-PE	ERP108802	Burns <i>et al.</i> (2018)
Adrenal cortex	2	PolyA-PE	ERP108802	Burns <i>et al.</i> (2018)
Cerebellum	3	Ribo-depleted-PE	SRP082342	Mansour <i>et al.</i> (2017)
Chorioallantois – 45 days	4	PolyA-PE	SRP127170	Dini <i>et al.</i> (2018)
Chorioallantois – 4 months	4	PolyA-PE	SRP219950	Loux <i>et al.</i> (2019b)
Chorioallantois – 6 months	4	PolyA-PE	SRP219950	Loux <i>et al.</i> (2019b)
Chorioallantois – 10 months	4	PolyA-PE	SRP219950	Loux <i>et al.</i> (2019b)
Chorioallantois – 11 months	4	PolyA-PE	SRP219950	Loux <i>et al.</i> (2019b)
Chorioallantois – post-partum	4	PolyA-PE	SRP219950	Loux <i>et al.</i> (2019b)
Chorionic girdle	4	cDNA-SE	SRP011379	Wang <i>et al.</i> (2012)
Endometrium – diestrus	4	PolyA-PE	SRP136284	Scaravaggi <i>et al.</i> (2019)
Inner cell mass (ICM)	3	cDNA-SE	SRP031504	Iqbal <i>et al.</i> (2014)
Kidney	3	cDNA-PE	SRP017611	Fushan <i>et al.</i> (2015)
Liver	2	PolyA-PE	ERP108802	Burns <i>et al.</i> (2018)
Lung	2	PolyA-PE	ERP108802	Burns <i>et al.</i> (2018)
Ovary	2	PolyA-PE	ERP108802	Burns <i>et al.</i> (2018)
Oviduct	20	PolyA-PE	SRP064530	Smits <i>et al.</i> (2016)
Skin	2	PolyA-PE	ERP108802	Burns <i>et al.</i> (2018)
Testes	2	PolyA-PE	SRP126383	Janečka <i>et al.</i> (2018)
Trophectoderm	3	cDNA-SE	SRP031504	Iqbal <i>et al.</i> (2014)

SRA, sequence read archive.

Table 2 Lineage-specific genes (ICM, TE, and PE) and ultimately which tissues they are expressed ('yes' = average TPM > 1). Modification of Fig. 4 of [Iqbal et al. \(2014\)](#), using our data reduction.

	Gene	TE	ICM	P-value*	CG	45 d	4 m	6 m	10 m	11 m	PP	EN	Oviduct	Skin	Lung	Kidney	Cerebellum	Adipose	Liver	Testes	Ovary	Adrenal
ICM	ENSECAG00000012614	NANOG	0.66	34.66	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	No	No
ICM	ENSECAG00000010653	SOX2	0.00	9.97	No	No	No	No	No	No	No	No	No	No	Yes	No	Yes	No	No	No	No	No
ICM	ENSECAG00000010613	KLF4	11.10	40.59	0	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
ICM	ENSECAG00000020994	LIN28B	0.60	4.80	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	No	No
ICM	ENSECAG00000012102	DNM1T3B	215.98	414.83	0.361	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
ICM	ENSECAG00000017191	SPP1	0.31	41.95	0.093	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
ICM	ENSECAG00000039888	UTF1	1.17	5.18	0.357	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
ICM	ENSECAG00000024187	SMARCA2	1.48	3.49	0.185	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
ICM	ENSECAG00000008738	ID2	57.03	204.39	0.096	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
ICM	ENSECAG00000013271	DPPA4	27.46	47.50	0.282	No	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	No	No
ICM	ENSECAG00000008967	POU5F1	122.04	206.00	0.277	No	Yes	Yes	Yes	Yes	No	Yes	Yes	No	Yes	No	Yes	Yes	No	Yes	Yes	Yes
TE	ENSECAG00000027754	CDX2	18.93	6.60	0	No	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
TE	ENSECAG00000011303	TEAD4	107.91	76.14	0.002	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
TE	ENSECAG00000016768	CATA2	100.72	40.81	0.025	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
TE	ENSECAG00000024574	GATA3	151.24	23.05	0	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes
TE	ENSECAG00000014608	ELF3	42.62	13.43	0.005	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	No	No	No
TE	ENSECAG00000020410	FREM2	13.40	6.46	0.008	No	Yes	No	No	No	No	Yes	Yes	Yes	No	Yes	No	No	No	No	No	Yes
TE	ENSECAG00000017468	TFAP2A	121.97	21.19	0.001	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	No	Yes	No	No
PE	ENSECAG00000020359	CATA4	9.89	21.78	0.299	No	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	Yes	Yes	Yes	No
PE	ENSECAG00000023279	CATA6	8.79	17.08	0.201	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
PE	ENSECAG00000032004	FOXA2	2.03	3.45	0.491	No	No	No	No	No	No	Yes	Yes	No	Yes	No	No	No	Yes	No	No	No
PE	ENSECAG00000024450	HNF4A	11.13	17.48	0.4	No	Yes	No	No	No	No	No	No	No	Yes	Yes	No	No	Yes	No	No	No
PE	ENSECAG00000017031	GDF3	0.06	7.11	0.078	No	No	No	No	No	No	No	No	No	No	No	No	Yes	No	Yes	No	No
PE	ENSECAG00000018176	PDCFRA	2.53	33.78	0.036	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

*Statistically significant values are in bold.
D, days; ICM, inner cell mass; m, months; PE, primitive endoderm; TE, trophectoderm.

that many of these markers, whilst enhanced, are not always unique to the trophoblast lineages (Fig. 1 and Table 2). *TEAD4*, *GATA2*, *ELF3* are also expressed in the endometrium and are thus unsuitable for differentiating between these two tissues. Two markers with the most enriched expression in placental tissues are *CDX2* and *TFAP2A*. *CDX2* is expressed in early TE (day 8) and chorionic girdle cells (days 35–36), but meta-analysis suggests not chorioallantois (45 day+) or other tissues, although it should be noted the meta-analysis did not include equine intestinal tissue known to express *CDX2* in other species. *CDX2* is a homeobox transcription factor that has been studied extensively in the context of its role in maintaining stemness of trophoblast stem cells. More recent work suggests that it also plays a role beyond these early events. In cows, *CDX2* maintains expression in trophoblast post-implantation to at least day 20 where it activates *IFNT* expression via binding to the proximal promoter and inducing histone acetylation (Ezashi & Imakawa 2017). In human placenta, *CDX2* expression in villous cytotrophoblast remains strong at week 5 of gestation at the level of the chorionic plate but is absent in trophoblast nuclei of syncytial trophoblast and proximal cell column trophoblast suggesting it is lost as differentiation progresses (Horii *et al.* 2016, Soncin *et al.* 2018). Indeed, *CDX2* expression drops linearly between week 5 and week 18, by which time there are very few positive cytotrophoblast (Horii *et al.* 2016). The role of *CDX2* in the equine placenta is not known, but given the expression pattern here restricted to TE and chorionic girdle tissue, both tissues possessing trophoblast with stem cell characteristics, it likely primarily relates to maintaining trophoblast stem cells.

TFAP2A was highly expressed in all placental tissues examined to date, with low expression in testes and kidney and moderate expression in skin (Table 2). Epigenetic markers such as *DNMT3B*, whilst highly expressed in ICM, are also abundant in TE (Iqbal *et al.* 2014, Table 2). *DNMT3B* is the major DNA methyltransferase regulating DNA methylation during development, so not surprisingly it is expressed in the yolk sac to at least 19 days (Gibson *et al.* 2017) and chorioallantois throughout gestation but minimally in adult tissues (Table 2).

Yolk Sac

The yolk sac first forms around day 8 when a layer of endoderm starts to underlay the TE resulting in the primitive bilaminar yolk sac wall (Figs 1 and 2). Around day 12, the mesoderm starts to migrate between these layers providing the connective tissue and vasculature to form the trilaminar yolk sac (Figs 1 and 2), which ultimately transitions to the vascular yolk sac between days 18 and 20, a process that is comprehensively described in previous works (Betteridge *et al.* 1982, Enders *et al.* 1993). There are no published studies of

global gene expression in either the bi- or tri-laminar yolk sac, although the transcriptome of whole conceptuses between days 8 and 14 has been reported (Klein & Troedsson 2011).

A number of small studies have investigated gene and protein expression of select molecules proposed to be important to yolk sac functions, though most do not differentiate between the bi- and tri-laminar layers due to difficulties with dissection. Examples of key genes expressed in the yolk sac include maternally (*GRB10*, *H19*, *IGF2R* and *PHLDA2*) and paternally (*DIO3*, *IGF2*, *INSR*, *PEG3*, *PEG10*, *NDN* and *SNRPN*) imprinted genes, epigenetic regulators such as DNA methyltransferases (*DNMTs*) and growth factors and related molecules (*VEGFA*, *FLT* and *KDR*) (Gibson *et al.* 2017). Many of these genes increase in expression in the developing yolk sac where they are likely to play important roles in sensing the environment and/or regulating the growth of the early embryo or yolk sac itself (Gibson *et al.* 2017). Yolk sac endoderm and trophoblast cells also express amino acid transporters such as *SLC2A1* (Gibson *et al.* 2018), a glucose transporter acting to nourish the earliest stages of embryonic development. By day 14–16, yolk sac is hormonally active, expressing a high level of P450 aromatase (*CYP19A1*; (Walters *et al.* 2000)), *ESR2* (Rambags *et al.* 2008) and *17B-HSD* (Raeside *et al.* 2009) as well as *PGF2A* and *PGE* (Stout & Allen 2002) and the oxytocin receptor (Budik *et al.* 2012) indicating the yolk sac trophoblast actively regulates oestrogen and prostaglandin metabolism and bioactivity.

Amniotic membrane

The amnion first develops around day 16, resulting from folding of the chorion (ectoderm and avascular mesoderm layers) around the embryo (Fig. 1). Fusion is complete by 20 days (Acker *et al.* 2001), immediately prior to the loss of the capsule. There has been interest in the amnion at term as it is commonly used for wound healing, including treatment of corneal ulceration (Lassaline *et al.* 2005). Although the mechanism of action is unclear, it has been proposed to act through stem cell-associated factors (Seo *et al.* 2013), and/or through microvesicles containing anti-inflammatory miRNAs, capable of modulating interleukin signalling (Lange-Consiglio *et al.* 2018). Whilst the proteome of amnion has been described, including lumican and dermatopontin which are believed to aid in corneal membrane healing (Galera *et al.* 2015), very little is otherwise known about amniotic gene expression.

Chorionic girdle

The chorionic girdle has been more extensively characterised than any of the other early equine placental membranes (Brosnahan *et al.* 2012, Wang *et al.* 2013,

Read *et al.* 2018a). At day 27, the tissue is initially composed of a single layer of vascular mesoderm cells which sit underneath a single layer of tall columnar trophoblast (Figs 1 and 2) (Antczak *et al.* 2013). In the ensuing days, the trophoblast cells start to rapidly proliferate (Gerstenberg *et al.* 1999) forming a stratified columnar epithelium which by day 34 involves multiple layers of trophoblast which are thrown into folds (Fig. 2). The trophoblast morphology undergoes extensive changes over this period converting from primarily uni-nucleate cells with a small cytoplasmic diameter to majority binucleate cells with a large cytoplasm (Antczak *et al.* 2013). Therefore, any study of gene changes in the chorionic girdle must be considered alongside these profound changes in tissue composition and will most certainly reflect the proportion of uni-/bi-nucleate cells present. Not surprisingly, this tissue transformation is accompanied by dynamic changes in the transcriptome of the chorionic girdle with 1625 genes differentially expressed during chorionic girdle development compared with the essentially unaltered gene profile of the adjacent chorion over the same 7-day period (Read *et al.* 2018a). The majority of the genes rapidly increase or decrease in expression over this period but of interest is a group of 83 genes that are transiently increased at days 30/31 (Read *et al.* 2018a) and may well be critical in controlling the rapid proliferation and differentiation of the tissue. These include spliceosome-related genes, as well as *FGF10*, a governor of trophoblast movement in ewes (Yang *et al.* 2011) and cell cycle regulators such as *CDC16*, an APC complex member that governs exit from mitosis.

The chorionic girdle distinguishes itself from other trophoblast tissues in its high expression of major histocompatibility complex (MHC) class I (Bacon *et al.* 2002), discussed in more detail below. For decades, chorionic girdle trophoblast has been thought of as an immunologically active tissue (Noronha & Antczak 2012) that stimulates antigen-specific B cell responses (Antczak & Allen 1989), attracts a T cell infiltration characterised by immunosuppressive Treg cells (de Mestre *et al.* 2010, Noronha & Antczak 2012) and modulates lymphocyte function (Flaminio & Antczak 2005). Indeed, chorionic girdle trophoblast can be transplanted across allogeneic barriers into the vulva of non-pregnant mares and survive for up to 3 months (de Mestre *et al.* 2011) as well as resist secondary immune responses (Brosnahan *et al.* 2016) further demonstrating the powerful immunological properties of the cells. Chorionic girdle trophoblast produces cytokines TNF (Grünig & Antczak 1995) and IL-22 (Brosnahan *et al.* 2012), the latter likely involved in mucosal immunity and repair of epithelia during the invasion process (Brosnahan *et al.* 2012). Chorionic girdle cells are also hormonally active; between days 27 and 34, there is a rapid and substantial upregulation of the chorionic gonadotrophin alpha and luteinizing hormone beta

(*LHB*) (previously *CGB*) subunits facilitating equine chorionic gonadotrophin (eCG) expression (de Mestre *et al.* 2009), ultimately comprising the first and seventh most abundant protein-coding genes in chorionic girdle respectively (Fig. 2). Expression of *LHB* is regulated directly by transcription factors *GCM1* and *ELF5*, which show a similarly dramatic upregulation (Read *et al.* 2018b).

Growth factors comprise another abundantly expressed group of genes in the chorionic girdle and are believed to act in an autocrine manner to coordinate the development of the chorionic girdle along with spatial coordination of adjacent tissues such as the allantochorion (Fig. 2). Read *et al.* (2018a) showed an upregulation of ERK1/2 activity in *ex vivo* day 30 chorionic girdle tissue suggesting this pathway is active corresponding to a period when *in vivo* trophoblast proliferation is maximal (Read *et al.* 2018a), although *in vivo* regulation of ERK1/2 and AKT1 pathways is not known. Curiously *HGF*, *EGF* and *FGF2* are not expressed in any appreciable quantities in chorionic girdle tissue or allantochorion between days 45 and term, although *EGF* is present in the adjacent endometrium and may be acting in a paracrine manner (Allen *et al.* 2017, de Ruijter-Villani *et al.* 2013). *FGF7* is one of the top ten most highly expressed genes in day 31 chorionic girdle trophoblast (Read *et al.* 2018a) with expression sustained at day 34, and coinciding with expression of its receptor, *FGFR2* (Read *et al.* 2018a). *IGF2* (and less so *IGF1*) and its receptors *IGF1R* and *IGF2R* along with a number of the insulin-like growth factor-binding proteins (IGFBPs) are highly expressed by both chorionic girdle tissue and allantochorion (Lennard *et al.* 1995; Fig. 2). Although placental IGF signalling is well defined in other species (Sferruzzi-Perri *et al.* 2017) where it drives proliferation and migration of primary trophoblast cells, functional studies are urgently needed to define the role of these placentally derived growth factors in equine placental development and expansion (Fig. 2).

Whilst mechanistic studies are lacking for these growth factors, TGF β ligand BMP4 has been shown to regulate differentiation of chorionic girdle trophoblast acting via SMAD1/5 (Cabrera-Sharp *et al.* 2014). BMP4 was found to be expressed by both the chorionic girdle itself and adjacent endometrium suggesting both autocrine and paracrine functions (Cabrera-Sharp *et al.* 2014) (Fig. 2). SMAD2 signalling is also active in primary chorionic girdle trophoblast, supporting the possible importance of other TGF β ligands in regulating early placental development (Cabrera-Sharp *et al.* 2014).

The binucleate terminally differentiated chorionic girdle cells dislodge from the conceptus around 36 days and remain viable in the endometrium until approximately 100 days. The exact mechanisms that result in the death of the endometrial cup trophoblast are not definitively shown, although it is assumed

to be due to either CD4 and CD8+ lymphocyte-mediated destruction or via pre-programmed cell death. Not surprisingly, the endometrial cup trophoblast shares expression of a number of genes with mature chorionic girdle cells, but equally, gene expression is also modulated by the new endometrial environment (Aleksic *et al.* 2016). The best characterised example is expression of MHC class I, which is downregulated in binucleate trophoblast following the establishment of the endometrial cups then subsequently upregulated late in the lifespan of the cells (Antczak & Allen 1989). Pregnancy-specific glycoproteins also show complex regulation between these two environments (Aleksic *et al.* 2016) with some pregnancy-specific glycoproteins genes exclusively expressed in chorionic girdle tissue (CEACAM44), the endometrial cups (CEACAM48) or both tissues (CEACAM49).

Chorioallantois (allantochorion)

The allantois originates from the posterior end of the embryonic gut and develops rapidly until it surrounds the embryo and fuses with the chorion to form the allantochorion that will become the main placenta in the mare (Fig. 1). At 45 days, the allantois has completely replaced the yolk sac, which has been incorporated into the elongating umbilical cord. The trophoblast cells of the newly formed allantochorion are cuboidal to low columnar (Fig. 2) and remain as a single layer of cells that are in close apposition with the endometrial cells with villi not forming until 50 days. Although little is known about global gene expression in the early stages of allantochorion formation, expression of growth factors and their receptors is well characterised, as in chorionic girdle. Allantochorion trophoblast expresses *EGFR* from as early as days 25–30 with expression dramatically upregulated by days 35–40 (Allen *et al.* 2017). Other growth factors *VEGFA*, *FLT1* and *KDR* are all expressed by days 25 and 38 (Allen *et al.* 2007), with expression maintained throughout gestation (Loux *et al.* 2019b). There is a predominance of VEGF family members in allantochorion (Loux *et al.* 2019b), with *VEGFA* showing significant expression even in 8 day TE (Iqbal *et al.* 2014) while *VEGFB* and *VEGFC* are expressed by 45 days. Of the growth factors studied, the IGF family is the most abundant in early gestation and is discussed further below.

Overall, the 45-day allantochorion shows signs of being in a transitory state between the largely self-reliant embryo and the mature fetoplacental unit. Meta-analysis revealed 376 genes are up-regulated more than 100-fold between 8-day TE and 45-day allantochorion, with 91% of these expressed at minimal levels in 8-day TE (< 1 TPM), confirming major regulatory changes during this period (Wang *et al.* 2012, Iqbal *et al.* 2014). In comparison, only 17 genes had 100-fold higher

expression at 4 months vs 45 days (Wang *et al.* 2012, Loux *et al.* 2019a). Although far more genes showed increasing expression from TE to 45-day allantochorion, a few genes declined significantly in this time frame. Among these, most noticeable is a steep decline in pluripotency factors *POU5F1*, *LIN28A* and *ESRRB* as the placenta begins to mature and stabilize. Furthermore, waning expression of metabolism-related genes (*MIOX*, *ACSL6*, *TTC39B*) is seen as implantation occurs and the embryo increasingly begins to rely more heavily on nutrients from maternal circulation. Lastly, multiple histone transcripts (*HIST2H2BF*, *HIST1H1A*, *HIST1H1B*, *HIST1H1E*) show decreasing expression, with novel transcripts and small RNAs such as *eca-miR-9173*, *snoRNA33* and *RF02271* making up the majority of remaining down-regulated transcripts.

Steroidogenesis-related transcripts tell a similar story of a transitioning tissue, as well as providing hints into tissue physiology. For example, the 45-day allantochorion produces high levels of the enzymes required for cholesterol synthesis as well as *STAR*, *CYP11A1* and *CYP17A1*, suggesting that, as in the rabbit, this early tissue is able to effectively synthesize cholesterol *de novo*, as well as mediate conversion to the $\Delta 5$ androgen DHEA (Loux *et al.* 2020). A strong correlation exists between these placental enzyme transcripts and DHEAS in maternal circulation ($r > 0.978$; $P < 0.001$; Legacki *et al.* 2019), lending credence to a placental origin for DHEAS. This is in contrast to later gestation (≥ 4 months) when DHEA synthesis switches primarily to the fetal gonad, corresponding with an increase in unsulfonated DHEA in maternal circulation (Legacki *et al.* 2017).

Enzymes present later in the steroidogenic pathway, including *HSD17B1*, *HSD17B2*, *HSD3B2* and *CYP19A1* are present across gestation, albeit at relatively lower levels in the 45-day allantochorion than later gestational ages (Loux *et al.* 2020). These data are consistent with the work from the groups of both Raeside and Sharp which found that equine embryos produce estrogens in extraembryonic membranes as well as in the ICM (Raeside *et al.* 2012) as early as 12 days of gestation (Zavy *et al.* 1984). As the fetoplacental unit continues to mature (45 days to 4 months), additional endocrine transcripts show significant up-regulation including *AKR1C1*, *AKR1C23-like*, *PTGDS*, *SULT1E1*, *OXTR*, *NR4A1*, *GSTA3* and *SULT1A1* (Loux *et al.* 2020).

The fetomaternal placental unit appears to be fully mature by 4-month gestation, although the exact point of maturation can only be defined as >45 days and ≤ 4 month gestation. Maturation here is defined by a lack of significant changes in gene expression (as compared to 6-month allantochorion); false discovery rate (FDR) P -value < 0.05; (Loux *et al.* 2019a)). These two periods also mark a significant shift in tissue structure: a single layer of trophoblast in apposition with endometrium is observed at around day 45 (Fig. 2), whilst by 4 months,

microcotyledons and villi have formed. Between 80 and 120 days is also the approximate time of the luteo-placental shift, when progesterone synthesis has shifted away from progesterone (P4) synthesis by the corpus lutea and moved primarily to 5 α -dihydroprogesterone (DHP) synthesis by the allantochorion (Conley & Ball 2019). By 10 months, gene expression changes are again becoming apparent, with transcription starting to shift towards parturition and away from steroidogenesis as noted by a decrease in intracellular cholesterol transport transcripts (*ABCA1*, *ABCG1*, *LRP6*, *OSBP*, *STAR*, *NPC1*, *NPC2*; (Loux et al. 2019a)) and steroidogenic enzymes themselves, most notably *CYP19A1* (Loux et al. 2020). Evidence for the balance of steroidogenesis between the allantochorion and endometrium is apparent at this time, as expression of most steroidogenic enzymes continues to increase in the allantochorion, while concurrently declining in the endometrium. This phenomenon is particularly pronounced for the $\Delta 4 \rightarrow \Delta 5$ steroidogenic enzymes *HSD17B1*, *HSD17B2*, *HSD3B2* and *GSTA3* but is also evident in downstream pathway enzymes such as 5 α -reductase *SRD5A1*, as well as *AKR1C1*, *SULT1E1* and *SULT1A1* (Loux et al. 2020).

Preliminary evidence suggests that gene expression levels may be both correlatory and predictive of function. In addition to the tight correlation between placental *CYP17A1* and maternal DHEAS described above, additional select transcripts were significantly correlated to their expected steroid product in maternal circulation (Fig. 3). For example, *CYP19A1*, the transcript for P450 aromatase, was correlated with maternal estrone sulphate levels ($r = 0.661$), while *SRD5A1* (5 α reductase) was correlated with maternal 5 α -dihydroprogesterone ($r = 0.552$).

Non-coding RNAs such as miRNAs are also indisputably important in allantochorion development. In early equine pregnancy, miRNA have been proposed to be involved in maternal recognition of pregnancy (Klohonatz et al. 2016). Orthologous members of the placental-specific C14MC cluster have been shown to be up-regulated in maternal circulation during pregnancy (Dini et al. 2019), with these and other miRNA shown to change significantly during late pregnancy and placental infections (Loux et al. 2017), with both tissue-level and circulatory miRNAs showing distinct expression patterns based on the stage of disease (Loux et al. 2019b).

Due to the bulk tissue approaches used to date for the above studies, remarkably we don't know yet how these changes relate to the marked change in tissue composition of the allantochorion across gestation, or whether any of these genes are trophoblast specific, or reflect changes to the phenotype of the trophoblast cells themselves. Application of single-cell transcriptomic assays is therefore a must if we are to tackle this complexity and identify the upstream regulators of normal allantochorion placentation in the mare.

Figure 3 Correlation between circulating steroids and placental gene expression. Concentration of steroids in maternal circulation is indicated by dashed grey line (---), while placental transcript levels are indicated by black circles (●). (A) Correlation of DHEA sulphate (DHEAS) to *CYP17A1*; (B) correlation of estrone sulphate to *CYP19A1* (aromatase); and (C) correlation of 5 α -dihydroprogesterone (DHP) to *SRD5A1* (5- α reductase 1). Serum steroid data obtained from Legacki et al. (2019) (DHEAS and E1S) and Legacki et al. (2016) (DHP). The 50-week time point represents post-partum serum samples. Error bars indicate S.E.M. All correlations were significant ($P < 0.001$).

Term placentae

Literature addressing gene expression changes at parturition is slim, likely due to the difficulty in obtaining late gestational samples for comparison

purposes. Further, most of what we have learnt about the term placenta has been gleaned from data on control placentae for pathologies such as retained fetal membranes (Jaworska *et al.* 2021). A full review of the most important genes found to be responsive to pathologies, environmental cues and mare factors can be found in Robles *et al.* (Robles *et al.* 2022).

There have been a few publications that have focused on gene expression in the non-pathological term placenta, such as work by Loux *et al.* (2020) which includes samples throughout gestation and postpartum, providing some insight into driving changes, including the endocrine changes discussed above (Fig. 3). In addition, we were able to use the serial data set from Loux *et al.* (2019a) to confirm and expand hypotheses from other works. Circadian rhythm genes (*CLOCK*, *CRY1*, *CRY2*, *PER1*, *PER2*) were identified in equine placenta by Parsons Aubone *et al.* (2020); however, they were not able to find a correlation between relative expression and gestational length. By using the serial data set, we were able to identify an increase in *CRY1*, *CRY2* and *PER1* during late gestation, with *PER1* increasing over 400% between 11 months and postpartum placenta. These data also confirmed strong expression of MHC-I genes at parturition (Rapacz-Leonard *et al.* 2018), with expression notable in allanto chorion from as early 4 months, albeit at all gestation ages at a level that remains well below that observed in the chorionic girdle. As well, it supported work by Oddsdóttir *et al.* (2011) finding an increase in *MMP2* and -9 transcript in the postpartum placenta compared with 11-month allanto chorion (Oddsdóttir *et al.* 2011).

High-impact placental genes

Genes that differentiate early placental tissues trophoderm, chorionic girdle and allanto chorion

Comparison of gene expression in placental lineages allows for identification of important candidate genes responsible for the development and function of individual tissues, but due to the large number of genes identified, it can be difficult to characterise the most essential. In early development, the TE contributes to the chorion trophoblast which in turn gives rise to both the chorionic girdle and allanto chorion. Ergo, we utilize the meta-analysis to identify a subset of predicted 'high-impact' genes to better define the transition from TE to allanto chorion and chorionic girdle. These genes were both highly expressed (TPM > 100) and highly up-regulated (fold change > 100) between the common derivative tissue, 8-day TE, and one or both of the two main tissue lineages of the equine placenta, chorionic girdle (day 33) and allanto chorion (45 days and 4 months). This identified a total of 182 genes, many of which showed significant changes in expression among multiple tissues and time points (Supplementary Table

1, see section on [supplementary materials](#) given at the end of this article). Notably, only half of these were identified as protein-coding genes, with the remainder classified as novel or ncRNA. This suggests that changes in ncRNA may be as critical to successful early embryonic development and placental differentiation as the better-studied changes in protein-coding genes, although it should be remembered that differences in library preparation between the TE and CG (cDNA, single-end reads) and allanto chorion tissues (Poly-A selection paired-end reads) may introduce bias. A direct comparison of gene expression between chorionic girdle and allanto chorion for a number of key placental processes reveals distinct roles, chorionic girdle predominantly enriched for gonadotrophin expression in line with its function of eCG production as well as genes involved in antigen presentation (Fig. 4), whereas allanto chorion showed relatively higher expression of angiogenic, chemotactic, extracellular matrix (ECM) and focal adhesion genes.

During the transition from 8-day TE to 45-day allanto chorion, most genes were up-regulated (Supplementary Table 1); however, there was a subgroup which was down-regulated, including pluripotency factors *POU5F1*, *ESRRG* and *LIN28A*. Amino acid transporter *SLC36A2*, known to be highly upregulated in equine endometrium in early pregnancy, was also down-regulated between TE and 45 days (Klein *et al.* 2010) possibly indicating a difference in embryonic metabolism. In addition to the increase in endocrine-related transcripts described earlier, there is a clear up-regulation in expression of ECM-related factors, including a number of collagen family members (Supplementary Tables 1 and 2). Remarkably, this dramatic collagen up-regulation seems to be much more prevalent in the 45-day allanto chorion than the chorionic girdle (Fig. 4), perhaps a reflection of the more temporary nature of the chorionic girdle cells. This discrepancy extends into other ECM-related transcripts including collagen fibril organizer, lumican (*LUM*) and embryo adhesion factor, thrombospondin (*THBS1*; (Kolakowska *et al.* 2017)). Fibrinogen has been suggested to be involved in embryonic adhesion in an integrin-dependent manner (Grant *et al.* 2020); this hypothesis is well supported by our data as we identified not only *FGB* but also many other focal adhesion and ECM proteins changing significantly here as well (Fig. 4).

In the transition from TE to chorionic girdle, a different pattern emerges. Although an up-regulation of both ECM and endocrine-related transcripts exists (Supplementary Table 1), the chorionic girdle cells also have an increase in transcripts related to ECM degradation (*MFAP2*) and autophagy (*RAB17*), presumably related to their invasive properties. Expression of the fetal haemoglobin subunit *HBE* was primarily within the allanto chorion throughout gestation, although transcription levels

Figure 4 Functional comparison of high-impact genes in the allantochorion (ALC) and chorionic girdle (CG). Inclusion criteria include >100x increase from 8-day TE and >100 TPM in either 45-day ALC or 34-day CG. Location along X-axis indicates relative fold-change between ALC and CG by category, with more distal localization indicating increasingly tissue-biased expression.

declined steadily until 11 months gestation. Of note, another fetal hemoglobin subunit (*HBZ*) showed the highest expression in chorionic girdle cells (Fig. 4 and Supplementary Table 2). Curiously, no expression of *HBZ* is seen in either allantochorion (> 4 months) or fetal liver. This differs significantly from humans where *HBZ* is produced in the fetal liver after ~8 weeks of gestation. The lack of *HBZ* production in equine fetal liver is likely to reflect species differences, as another fetal liver gene, *AFP*, is transcribed in the early placenta (chorionic girdle and 45-day allantochorion), then shifts to the liver by 4 months gestation, similar to humans.

A number of apolipoproteins were identified as high-impact genes in early development, including *APOA1*,

APOC2, *APOE* and *APOM* (Supplementary Table 1). Best known for their role in cholesterol transport, apolipoproteins have been shown to have a protective, anti-inflammatory effect in human placenta and may act to prime maternal endothelial cells for trophoblastic invasion (Charlton *et al.* 2017). Equine *APOA1* and *APOE* are highly expressed in the early allantochorion and also found in chorionic girdle, with the equine allantochorion also expressing high levels of *APOD* throughout pregnancy. A subset of chorionic girdle and allantochorion samples (35–45 days) were found to produce an even wider range of apolipoproteins, including *APOC2* and *APOM* (Fig. 4).

The IGF family plays a prominent role in early gestation; IGF2 is the most abundant growth factor in both chorionic girdle and allantochorion (45 days) and the only growth factor to be included in Supplementary Table 2. Several other IGF family members only fell just short of the 100 TPM threshold, including *IGF2R* and *IGFBP2*, 3, 5 and 7, making this family the most abundant in early equine placental tissue with expression levels increasing 50- to 6000-fold over 8-day TE. As in humans and mice, equine *IGF2* is a paternally imprinted gene best known for driving fetal growth, while its receptor, *IGF2R* is maternally imprinted (Wang *et al.* 2013). However, a recent equine study (Dini *et al.* 2021) found that *IGF1*, *IGF1R* and *INSR* are also paternally imprinted, while *IGFBP4*, a negative regulator of angiogenesis (Contois *et al.* 2012), is maternally expressed, speaking to a delicate balance of maternal and paternal regulation. IGFs also appear to be environmentally regulated; during negative asynchronous embryo transfer, extra-embryonic expression is delayed but not dysregulated, a feature proposed to contribute to the resilience of equine embryos in an asynchronous uterine environment (Gibson *et al.* 2017). Whilst functional studies are lacking, IGF1 protein expression has been shown in microcotyledons at day 130 of pregnancy with expression decreasing over gestation and positively correlating with the number of proliferating trophoblast suggesting a possible role in promoting allantochorion and more specifically microcotyledonary development (Arai *et al.* 2006).

Next, we aimed to identify specific gene markers of differentiation that differed between allantochorion and chorionic girdle cells by identifying genes with >100 fold-change and >10 TPM in the tissue of interest. In total, there were 199 genes with enriched expression in chorionic girdle cells and another 80 with enriched expression in 45-day allantochorion (Supplementary Table 2). Although the majority of chorionic girdle genes were either novel genes or ncRNA, there were three protein-coding genes which had exclusive expression in chorionic girdle cells: *FABP4*, *FXD6* and *PXMP2*. *FABP4* is the most highly upregulated gene in both day 30 and 31 chorionic girdle (Read *et al.* 2018a), suggesting expression is sustained for a good proportion of chorionic

Figure 5 Expression of 42/77 high-impact placenta-enriched genes as defined as TPM < 1 in non-placental tissues and TPM > 10 in placental tissue(s). Trophoblast (TE), inner cell mass (ICM), chorionic girdle (CG) and allantochorion between 45 days and 11 months, postpartum (PP) chorioallantois and diestrus endometrium (EN). Expression of an additional 35/77 transcripts with no assigned gene can be found in Supplementary Fig. 1.

girdle development. It is an excellent candidate gene to drive development of the chorionic girdle, having been found to promote trophoblast proliferation, migration and invasion in human trophoblast (Yan *et al.* 2016). The smaller total number of identified ALC-enriched genes contained a much higher number of protein-coding genes ($n=54$), including *SPAM1*, *LTF* and *HPGD*, among others (Supplementary Table 2). We propose that these genes may be used as lineage-specific markers to differentiate between these two closely integrated tissue lineages.

Placental-enriched transcripts across pregnancy

The placenta is a rapidly forming and expanding organ with diverse physiological functions, so it is not surprising that equine placental tissue expresses an array of genes in common with other tissues and is normally associated with processes such as epigenetic regulation, cell proliferation, differentiation, immunity and movement. Indeed, it is a commonly held view across species that the placenta expresses very few placental-specific genes

as they would be a target for immunological attack by the mother's immune system. A anti-trophoblast antibody (102.1) that labels equine allantochorion and chorionic girdle trophoblast cells between day 12 and term has been widely utilized by researchers since the initial report (Oriol *et al.* 1993). The antibody labels an unknown antigen, with the original study suggesting it may be trophoblast specific as the antibody failed to label other cells in day 8 embryos as well as all 18 adult and 6 non-placental fetal tissues assessed (Oriol *et al.* 1993). There were two transcripts in our metanalysis that had an expression profile across placental and non-placental tissues that best matched this antigen distribution: Claudin 1 (CLDN1) and Iroquois-class homeodomain protein 4 (IRX4). IRX4 is a transcription factor with primarily nuclear expression in tumour and cardiac cells so is unlikely to represent the antigen detected by 102.1 which is mostly localized to the cell surface. Claudin 1 remains a strong candidate. It is highly expressed in human chorionic villous trophoblast (Zhang *et al.* 2021) and as a membrane protein and major contributor to tight junction complexes, it shows similar distribution

to the antigen labelled by 102.1. Further work must be done to verify this hypothesis.

Lastly, to identify further genes of importance, we began by looking for placental-enriched genes (>10 TPM in placental tissue(s), <1 TPM in non-placental tissues) with no fold-change requirement, ultimately identifying 71 genes (Fig. 5). Four enriched genes (*IGF2BP3*, Zinc Finger Protein 42 (*ZFP42*), Vestigial Like Family Member 1 (*VGLL1*), Paired Like Homeodomain 1 (*PITX1*)) are expressed in all placental tissues examined to date (TE, chorionic girdle, allantochorion). IGF family member *IGF2BP3* was predictably enriched, as the IGF system is known to be highly active in early placental development in many species (Sferruzzi-Perri *et al.* 2017), including equine as discussed above. Another placentally enriched gene, *VGLL1* is a TEA family transcription factor found to have sustained co-expression with *TEAD4* across gestation in human trophoblast, though *VGLL1* appears absent in murine placenta (Soncin *et al.* 2018). The sustained expression of *VGLL1* in equine placental tissues suggests the gene is not human-specific, highlighting the necessity of utilising comparative animal models of early placental development to complement work done in mice. *ZFP42*, aka Rex1, has long been used as a marker of pluripotent murine embryonic stem cells, although does not appear to be required for pluripotency itself (Masui *et al.* 2008). Expression patterns in equine placenta suggest a similar specificity and function. Lastly, *PITX1*, a GCM1 binding partner is enhanced across gestation (discussed further below; Read *et al.* 2018b).

A further four placental-enriched genes were expressed in all placental tissues except day 8 TE (*HAND1*, *TBX20*, *ANKRD55*, *GDF6*). *HAND1* is a well-characterised placental and cardiac transcription factor previously described in chorionic girdle and allantochorion (de Mestre *et al.* 2009) and also identified as a 'early developmental high-impact' gene when comparing just the three early placental tissues, TE, chorionic girdle and allantochorion (Supplementary Tables 1 and 2). In mouse, *HAND1*-null animals have abnormalities in both trophoblast giant cell differentiation as well as cardiac development (Riley *et al.* 1998). Curiously, another of these enriched genes, *TBX20*, is a transcription factor primarily known for its role in cardiac development (Ahn *et al.* 2000); however, *TBX20* has not yet been studied in the placenta of any species. In the mouse, *Hand1* works in conjunction with other transcription factors including *Stra13* and *Gcm1* to promote trophoblast differentiation, albeit towards different fates. While all three transcription factors drive cell-cycle exit, *Hand1* and *Stra13* drive towards the giant trophoblast phenotype, while *Gcm1* is required for syncytiotrophoblast formation in the labyrinth region (Hughes *et al.* 2004). *STRA13* (*CENPX*) is expressed at minimal levels in equine placenta and does not meet our definition of placental-enriched gene.

The transcription factor, *GCM1*, is rapidly induced in the chorionic girdle between days 27 and 34 (de Mestre *et al.* 2009, Read *et al.* 2018b), with maximal mRNA expression seen in the allantochorion from 4 months onwards. It is known to regulate eCG via direct binding to the LHB promoter (Read *et al.* 2018b) and possesses multiple functions in human trophoblast, suggesting further function in equine trophoblast are likely to exist as well. *GCM1* protein expression is strong in the recently formed allantochorion (Read *et al.* 2018b), and gene expression persists in the allantochorion throughout gestation with the highest levels noted in term and post-partum allantochorion (Loux *et al.* 2019a) (Fig. 5). Due to the expression of *GCM1* across a number of placental compartments, it is likely that its function is tightly controlled and at times modulated by the co-expression of *GCM1* binding partners such as *PITX1* and *HOXA13* (Jolma *et al.* 2015), both also identified here as enriched genes (Fig. 5). *PITX1* expression levels are negatively associated with *GCM1*: they decrease during chorionic girdle development right as *GCM1* expression rises (Read *et al.* 2018b). This pattern is similarly observed in the allantochorion as *GCM1* expression is maximal in post-partum allantochorion, while *PITX* is simultaneously decreased to its lowest levels (Loux *et al.* 2019a). These changes appear placental-specific as a number of other *GCM1* binding partners are absent/show very low expression levels in equine placental tissues (*ONECUT2*, *HOXA2*, *FIGLA*, *SPDEF*, *SOX2*) (Loux *et al.* 2019a).

Conclusion and final remarks

Since the equine genome was sequenced in 2007, remarkable progress has been made in describing the genetic landscape of the maternal–fetal interface in the horse. Despite these numerous studies, it is clear that nearly all have used a bulk tissue approach so important questions remain regarding whether placental gene changes are reflective of changes in tissue composition and/or induction of genes in specific cells such as trophoblast. Further, full characterisation of equine trophoblast subtypes remains elusive. Moving forward, single-cell transcriptomics is pertinent if we are to appreciate the molecular events at the equine maternal–fetal interface and to be able to address questions such as: what genes mark uninucleate vs binucleate cells in the chorionic girdle? Does eCG expression directly follow acquisition of binucleate morphology or are these decoupled events? What genes drive differentiation of trophoblast in the allantochorion?

Bulk tissue transcriptomics revealed both conserved and unique genes likely important in regulating equine placental development and function; however, significant gaps remain in our knowledge of the functional relevance of most of the genes and this must be a priority of the next decade. Exactly which of these genes and pathways will yield the most fruitful results is

a difficult question. In order to address this, alongside the wider literature, we have looked holistically at all published transcriptomes of equine fetal membranes/tissues to generate a more focused list of genes that act as signatures of the three key fetal tissues in early placental development (TE, chorionic girdle and allantochorion), as well as those that are highly enriched in the placenta compared to eleven other equine tissues. Whilst the strict criteria applied will have undoubtedly meant some relevant genes will have been missed, the approach has provided a rich list of genes and gene families that warrant functional interrogation.

Wider researchers will be able to use this resource as a reference for designing future experiments, with certain genes and gene families highlighted as excellent candidates for more immediate mechanistic studies. For example, GCM1 is best known as a key gene of chorionic girdle tissue and regulator of CGB expression; however, our meta-analysis found it is widely expressed in equine chorioallantois and indeed the highest expression was found in term placenta. The function of GCM1 in chorioallantois is not known, although this expression along with its role in pathologies of pregnancy in other species suggests it should be a priority in any future studies. FABP4 was identified in meta-analysis as a CG-specific gene consistent too with previous data that found it to be the most highly expressed gene in days 30 and 31 chorionic girdle suggesting it has wide applications as a marker across chorionic girdle development with a functional role as yet undefined. Apolipoproteins were identified as high-impact genes of particular interest with dynamic expression of family membranes in early allantochorion, chorionic girdle or both. Shared expression of *VGLL1* between horse and human but not rodent placenta is a reminder of the complex evolution of placental trophoblast cells.

These gene lists need to be supplemented with a greater understanding of the epigenetic landscape and interpreted alongside future proteomics and secretomic studies. Such studies are now key not only to inform researchers of key pathways relevant to understanding the complexity of normal placental development and its role supporting fetal health but also to unravelling novel targets that are modified by the environment and play a role in pregnancy pathologies (Robles *et al.* 2022).

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-21-0115>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding

A M d M has received funding for work discussed in this review from Wellcome Trust, Alborada Trust, Thoroughbred Breeders Association. S L received funding for work discussed in this review from Kentucky Thoroughbred Owners and Breeders Association. P C P received funding for work discussed in this review from Institut Français du Cheval et de l'Équitation and INRAE for PhD salary support.

Author contribution statement

All authors contributed to the conceptualization of the review, S L analysed the data, S L and A d M prepared the figures, tables and the first draft of the manuscript and all authors reviewed and edited the manuscript.

Acknowledgements

We would like to thank Elaine Shervill, Anne Kahler and Jessica Roach for assistance with collection and/or sectioning and staining of tissue sections.

References

- Acker DA, Curran S, Bersu ET & Ginther OJ 2001 Morphologic stages of the equine embryo proper on days 17 to 40 after ovulation. *American Journal of Veterinary Research* **62** 1358–1364. (<https://doi.org/10.2460/ajvr.2001.62.1358>)
- Ahn DG, Ruvinsky I, Oates AC, Silver LM & Ho RK 2000 *tbx20*, a new vertebrate T-box gene expressed in the cranial motor neurons and developing cardiovascular structures in zebrafish. *Mechanisms of Development* **95** 253–258. ([https://doi.org/10.1016/S0925-4773\(00\)00346-4](https://doi.org/10.1016/S0925-4773(00)00346-4))
- Aleksic D, Blaschke L, Mißbach S, Hänse J, Weiß W, Handler J, Zimmermann W, Cabrera-Sharp V, Read JE, Mestre AM *et al.* 2016 Convergent evolution of pregnancy-specific glycoproteins in human and horse. *Reproduction* **152** 171–184. (<https://doi.org/10.1530/REP-16-0236>)
- Allen WR, Gower S & Wilsher S 2007 Immunohistochemical localization of vascular endothelial growth factor (VEGF) and its two receptors (Flt-1 and KDR) in the endometrium and placenta of the mare during the oestrous cycle and pregnancy. *Reproduction in Domestic Animals* **42** 516–526. (<https://doi.org/10.1111/j.1439-0531.2006.00815.x>)
- Allen WRT, Gower S & Wilsher S 2017 Localisation of epidermal growth factor (EGF), its specific receptor (EGF-R) and aromatase at the materno-fetal interface during placentation in the pregnant mare. *Placenta* **50** 53–59. (<https://doi.org/10.1016/j.placenta.2016.12.024>)
- Antczak DF & Allen WR 1989 Maternal immunological recognition of pregnancy in equids. *Journal of Reproduction and Fertility: Supplement* **37** 69–78.
- Antczak DF, de Mestre AM, Wilsher S & Allen WR 2013 The equine endometrial cup reaction: a fetomaternal signal of significance. *Annual Review of Animal Biosciences* **1** 419–442. (<https://doi.org/10.1146/annurev-animal-031412-103703>)
- Arai KY, Tanaka Y, Taniyama H, Tsunoda N, Nambo Y, Nagamine N, Watanabe G & Taya K 2006 Expression of inhibins, activins, insulin-like growth factor-I and steroidogenic enzymes in the equine placenta. *Domestic Animal Endocrinology* **31** 19–34. (<https://doi.org/10.1016/j.domaniend.2005.09.005>)
- Bacon SJ, Ellis SA & Antczak DF 2002 Control of expression of major histocompatibility complex genes in horse trophoblast. *Biology of Reproduction* **66** 1612–1620. (<https://doi.org/10.1095/biolreprod66.6.1612>)
- Betteridge KJ, Eaglesome MD, Mitchell D, Flood PF & Beriault R 1982 Development of horse embryos up to twenty two days after ovulation: observations on fresh specimens. *Journal of Anatomy* **135** 191–209.

- Brosnahan MM, Miller DC, Adams M & Antczak DF 2012 IL-22 is expressed by the invasive trophoblast of the equine (*Equus caballus*) chorionic girdle. *Journal of Immunology* **188** 4181–4187. (<https://doi.org/10.4049/jimmunol.1103509>)
- Brosnahan MM, Silvela EJ, Crumb J, Miller DC, Erb HN & Antczak DF 2016 Ectopic trophoblast allografts in the horse resist destruction by secondary immune responses. *Biology of Reproduction* **95** 135. (<https://doi.org/10.1095/biolreprod.115.137851>)
- Budik S, Palm F, Walter I, Helmreich M & Aurich C 2012 Increasing expression of oxytocin and vasopressin receptors in the equine conceptus between days 10 and 16 of pregnancy. *Reproduction, Fertility, and Development* **24** 641–648. (<https://doi.org/10.1071/RD11167>)
- Burns EN, Bordbari MH, Mienaltowski MJ, Affolter VK, Barro MV, Gianino F, Gianino G, Giulotto E, Kalbfleisch TS, Katzman SA *et al.* 2018 Generation of an equine biobank to be used for functional annotation of animal genomes project. *Animal Genetics* **49** 564–570. (<https://doi.org/10.1111/age.12717>)
- Cabrera-Sharp V, Read JE, Richardson S, Kowalski AA, Antczak DF, Cartwright JE, Mukherjee A & de Mestre AM 2014 SMAD1/5 signaling in the early equine placenta regulates trophoblast differentiation and chorionic gonadotropin secretion. *Endocrinology* **155** 3054–3064. (<https://doi.org/10.1210/en.2013-2116>)
- Charlton F, Bobek G, Stait-Gardner T, Price WS, Mirabito Colafella KM, Xu B, Makris A, Rye KA & Hennessy A 2017 The protective effect of apolipoprotein in models of trophoblast invasion and preeclampsia. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* **312** R40–R48. (<https://doi.org/10.1152/ajpregu.00331.2016>)
- Choi YH, Harding HD, Hartman DL, Obermiller AD, Kurosaka S, McLaughlin KJ & Hinrichs K 2009 The uterine environment modulates trophoblast POU5F1 levels in equine blastocysts. *Reproduction* **138** 589–599. (<https://doi.org/10.1530/REP-08-0394>)
- Conley AJ & Ball BA 2019 Steroids in the establishment and maintenance of pregnancy and at parturition in the mare. *Reproduction* **158** R197–R208. (<https://doi.org/10.1530/REP-19-0179>)
- Contois LW, Nugent DP, Caron JM, Cretu A, Tweedie E, Akalu A, Liebes L, Friesel R, Rosen C, Vary C *et al.* 2012 Insulin-like growth factor binding protein-4 differentially inhibits growth factor-induced angiogenesis. *Journal of Biological Chemistry* **287** 1779–1789. (<https://doi.org/10.1074/jbc.M111.267732>)
- de Mestre AM, Miller D, Roberson MS, Liford J, Chizmar LC, McLaughlin KE & Antczak DF 2009 Glial cells missing homologue 1 is induced in differentiating equine chorionic girdle trophoblast cells. *Biology of Reproduction* **80** 227–234. (<https://doi.org/10.1095/biolreprod.108.070920>)
- de Mestre A, Noronha L, Wagner B & Antczak DF 2010 Split immunological tolerance to trophoblast. *International Journal of Developmental Biology* **54** 445–455. (<https://doi.org/10.1387/ijdb.082795ad>)
- de Mestre AM, Hanlon D, Adams AP, Runcan E, Leadbeater JC, Erb HN, Costa CC, Miller D, Allen WR & Antczak DF 2011 Functions of ectopically transplanted invasive horse trophoblast. *Reproduction* **141** 849–856. (<https://doi.org/10.1530/REP-10-0462>)
- de Ruijter-Villani M, van Boxtel PRM & Stout TAE 2013 Fibroblast growth factor-2 expression in the preimplantation equine conceptus and endometrium of pregnant and cyclic mares. *Theriogenology* **80** 979–989. (<https://doi.org/10.1016/j.theriogenology.2013.07.024>)
- Dini P, Daels P, Loux SC, Esteller-Vico A, Carossino M, Scoggin KE & Ball BA 2018 Kinetics of the chromosome 14 microRNA cluster ortholog and its potential role during placental development in the pregnant mare. *BMC Genomics* **19** 954. (<https://doi.org/10.1186/s12864-018-5341-2>)
- Dini P, El-Sheikh Ali H, Carossino M, C. Loux S, Esteller-Vico A, E. Scoggin K, Daels P & Ball BA 2019 Expression profile of the chromosome 14 microRNA cluster (C14MC) ortholog in equine maternal circulation throughout pregnancy and its potential implications. *International Journal of Molecular Sciences* **20** 6285. (<https://doi.org/10.3390/ijms20246285>)
- Dini P, Kalbfleisch T, Uribe-Salazar JM, Carossino M, Ali HE, Loux SC, Esteller-Vico A, Norris JK, Anand L, Scoggin KE *et al.* 2021 Parental bias in expression and interaction of genes in the equine placenta. *PNAS* **118** e2006474118. (<https://doi.org/10.1073/pnas.2006474118>)
- Enders AC, Schlafke S, Lantz KC & Liu IKM 1993 Endoderm cells of the equine yolk sac from day 7 until formation of the definitive yolk sac placenta. *Equine Veterinary Journal* **25** 3–9. (<https://doi.org/10.1111/j.2042-3306.1993.tb04814.x>)
- Ezashi T & Imakawa K 2017 Transcriptional control of *IFNT* expression. *Reproduction* **154** F21–F31. (<https://doi.org/10.1530/REP-17-0330>)
- Flaminio MJBF & Antczak DF 2005 Inhibition of lymphocyte proliferation and activation: a mechanism used by equine invasive trophoblast to escape the maternal immune response. *Placenta* **26** 148–159. (<https://doi.org/10.1016/j.placenta.2004.05.008>)
- Fushan AA, Turanov AA, Lee SG, Kim EB, Lobanov AV, Yim SH, Buffenstein R, Lee SR, Chang KT, Rhee H *et al.* 2015 Gene expression defines natural changes in mammalian lifespan. *Aging Cell* **14** 352–365. (<https://doi.org/10.1111/ace.12283>)
- Galera PD, Ribeiro CR, Sapp HL, Coleman J, Fontes W & Brooks DE 2015 Proteomic analysis of equine amniotic membrane: characterization of proteins. *Veterinary Ophthalmology* **18** 198–209. (<https://doi.org/10.1111/vop.12190>)
- Gerstenberg C, Allen WR & Stewart F 1999 Cell proliferation patterns during development of the equine placenta. *Journal of Reproduction and Fertility* **117** 143–152. (<https://doi.org/10.1530/jrf.0.1170143>)
- Gibson C, de Ruijter-Villani M & Stout TAE 2017 Negative uterine asynchrony retards early equine conceptus development and upregulation of placental imprinted genes. *Placenta* **57** 175–182. (<https://doi.org/10.1016/j.placenta.2017.07.007>)
- Gibson C, de Ruijter-Villani M, Rietveld J & Stout TAE 2018 Expression of glucose transporters in the endometrium and early conceptus membranes of the horse. *Placenta* **68** 23–32. (<https://doi.org/10.1016/j.placenta.2018.06.308>)
- Grant DM, Macedo A, Toms D & Klein C 2020 Fibrinogen in equine pregnancy as a mediator of cell adhesion, an epigenetic and functional investigation. *Biology of Reproduction* **102** 170–184. (<https://doi.org/10.1093/biolre/iaz157>)
- Grünig G & Antczak DF 1995 Horse trophoblasts produce tumor necrosis factor α but not interleukin 2, interleukin 4, or interferon γ . *Biology of Reproduction* **52** 531–539. (<https://doi.org/10.1095/biolreprod52.3.531>)
- Horii M, Li Y, Wakeland AK, Pizzo DP, Nelson KK, Sabatini K, Laurent LC, Liu Y & Parast MM 2016 Human pluripotent stem cells as a model of trophoblast differentiation in both normal development and disease. *PNAS* **113** E3882–E3891. (<https://doi.org/10.1073/pnas.1604747113>)
- Hughes M, Dobric N, Scott IC, Su L, Starovic M, St-Pierre B, Egan SE, Kingdom JCP & Cross JC 2004 The Hand1, Stra13 and Gcm1 transcription factors override FGF signaling to promote terminal differentiation of trophoblast stem cells. *Developmental Biology* **271** 26–37. (<https://doi.org/10.1016/j.ydbio.2004.03.029>)
- Iqbal K, Chitwood JL, Meyers-Brown GA, Roser JF & Ross PJ 2014 RNA-Seq transcriptome profiling of equine inner cell mass and trophoblast. *Biology of Reproduction* **90** 61. (<https://doi.org/10.1095/biolreprod.113.113928>)
- Janečka JE, Davis BW, Ghosh S, Paria N, Das PJ, Orlando L, Schubert M, Nielsen MK, Stout TAE, Brashear W *et al.* 2018 Horse Y chromosome assembly displays unique evolutionary features and putative stallion fertility genes. *Nature Communications* **9** 2945. (<https://doi.org/10.1038/s41467-018-05290-6>)
- Jaworska J, Ropka-Molik K, Piórkowska K, Szmatoła T, Kowalczyk-Zięba I, Woławek-Potocka I & Siemieniuch M 2021. Transcriptome Profiling of the Retained Fetal Membranes—An Insight in the Possible Pathogenesis of the Disease. *Animals* **11** 675. (<https://doi.org/10.3390/ani11030675>)
- Jolma A, Yin Y, Nitta KR, Dave K, Popov A, Taipale M, Enge M, Kivioja T, Morgunova E & Taipale J 2015 DNA-dependent formation of transcription factor pairs alters their binding specificity. *Nature* **527** 384–388. (<https://doi.org/10.1038/nature15518>)
- Kalbfleisch TS, Rice ES, DePriest MS, Walenz BP, Hestand MS, Vermeesch JR, O'Connell BL, Fiddes IT, Vershinina AO, Saremi NF *et al.* 2018 Improved reference genome for the domestic horse increases assembly contiguity and composition. *Communications Biology* **1** 197. (<https://doi.org/10.1038/s42003-018-0199-z>)
- Klein C & Troedsson MHT 2011 Transcriptional profiling of equine conceptuses reveals new aspects of embryo-maternal communication in the horse. *Biology of Reproduction* **84** 872–885. (<https://doi.org/10.1095/biolreprod.110.088732>)
- Klein C, Scoggin KE, Ealy AD & Troedsson MHT 2010 Transcriptional profiling of equine endometrium during the time of maternal recognition

- of pregnancy. *Biology of Reproduction* **83** 102–113. (<https://doi.org/10.1095/biolreprod.109.081612>)
- Klohonatz KM, Cameron AD, Hergenreder JR, da Silveira JC, Belk AD, Veeramachaneni DN, Bouma GJ & Bruemmer JE 2016 Circulating miRNAs as potential alternative cell signaling associated with maternal recognition of pregnancy in the mare. *Biology of Reproduction* **95** 124. (<https://doi.org/10.1095/biolreprod.116.142935>)
- Kolakowska J, Souchelnytskyi S, Saini RKR & Franczak A 2017 Proteomic analysis of the endometrium during early pregnancy in the domestic pig. *Reproduction, Fertility, and Development* **29** 2255–2268. (<https://doi.org/10.1071/RD16435>)
- Lange-Consiglio A, Lazzari B, Perrini C, Pizzi F, Stella A, Cremonesi F & Capra E 2018 MicroRNAs of equine amniotic mesenchymal cell-derived microvesicles and their involvement in anti-inflammatory processes. *Cell Transplantation* **27** 45–54. (<https://doi.org/10.1177/0963689717724796>)
- Lassaline ME, Brooks DE, Ollivier FJ, Komaromy AM, Kallberg ME & Gelatt KN 2005 Equine amniotic membrane transplantation for corneal ulceration and keratomalacia in three horses. *Veterinary Ophthalmology* **8** 311–317. (<https://doi.org/10.1111/j.1463-5224.2005.00405.x>)
- Legacki EL, Scholtz EL, Ball BA, Stanley SD, Berger T & Conley AJ 2016 The dynamic steroid landscape of equine pregnancy mapped by mass spectrometry. *Reproduction* **151** 421–430. (<https://doi.org/10.1530/REP-15-0547>)
- Legacki EL, Ball BA, Corbin CJ, Loux SC, Scoggin KE, Stanley SD & Conley AJ 2017 Equine fetal adrenal, gonadal and placental steroidogenesis. *Reproduction* **154** 445–454. (<https://doi.org/10.1530/REP-17-0239>)
- Legacki EL, Scholtz EL, Ball BA, Esteller-Vico A, Stanley SD & Conley AJ 2019 Concentrations of sulphated estrone, estradiol and dehydroepiandrosterone measured by mass spectrometry in pregnant mares. *Equine Veterinary Journal* **51** 802–808. (<https://doi.org/10.1111/evj.13109>)
- Lennard SN, Stewart F & Allen WR 1995 Insulin-like growth factor II gene expression in the fetus and placenta of the horse during the first half of gestation. *Journal of Reproduction and Fertility* **103** 169–179. (<https://doi.org/10.1530/jrf.0.1030169>)
- Loux SC, Scoggin KE, Bruemmer JE, Canisso IF, Troedsson MH, Squires EL & Ball BA 2017 Evaluation of circulating miRNAs during late pregnancy in the mare. *PLoS ONE* **12** e0175045. (<https://doi.org/10.1371/journal.pone.0175045>)
- Loux SC, Dini P, El-Sheikh Ali H, Kalbfleisch T & Ball BA 2019a Characterization of the placental transcriptome through mid to late gestation in the mare. *PLoS ONE* **14** e0224497. (<https://doi.org/10.1371/journal.pone.0224497>)
- Loux SC, Fernandes CB, Dini P, Wang K, Wu X, Baxter D, Scoggin KE, Troedsson MHT, Squires EL & Ball BA 2019b Small RNA (sRNA) expression in the chorioallantois, endometrium and serum of mares following experimental induction of placentitis. *Reproduction, Fertility, and Development* **31** 1144–1156. (<https://doi.org/10.1071/RD18400>)
- Loux SC, Conley AJ, Scoggin KE, El-Sheikh Ali H, Dini P & Ball BA 2020 New insights in equine steroidogenesis: an in-depth look at steroid signaling in the placenta. *Reproduction* **160** 65–82. (<https://doi.org/10.1530/REP-20-0015>)
- Mansour TA, Scott EY, Finno CJ, Bellone RR, Mienaltowski MJ, Penedo MC, Ross PJ, Valberg SJ, Murray JD & Brown CT 2017 Tissue resolved, gene structure refined equine transcriptome. *BMC Genomics* **18** 103. (<https://doi.org/10.1186/s12864-016-3451-2>)
- Masui S, Ohtsuka S, Yagi R, Takahashi K, Ko MSH & Niwa H 2008 Rex1/Zfp42 is dispensable for pluripotency in mouse ES cells. *BMC Developmental Biology* **8** 45. (<https://doi.org/10.1186/1471-213X-8-45>)
- Noronha LE & Antczak DF 2012 Modulation of T-cell reactivity during equine pregnancy is antigen independent. *American Journal of Reproductive Immunology* **68** 107–115. (<https://doi.org/10.1111/j.1600-0897.2012.01154.x>)
- Noronha LE, Huggler KE, de Mestre AM, Miller DC & Antczak DF 2012 Molecular evidence for natural killer-like cells in equine endometrial cups. *Placenta* **33** 379–386. (<https://doi.org/10.1016/j.placenta.2012.01.018>)
- Oddsóttir R, Riley SC, Leask R, Shaw DJ, Aurich C, Palm F, Fowden AL, Ricketts SW & Watson ED 2011 Dynamics of activities of matrix metalloproteinases-9 and -2, and the tissue inhibitors of MMPs in fetal fluid compartments during gestation and at parturition in the mare. *Theriogenology* **75** 1130–1138. (<https://doi.org/10.1016/j.theriogenology.2010.11.023>)
- Oriol JG, Betteridge KJ, Hardy J & Sharom FJ 1993 Structural and developmental relationship between capsular glycoproteins of the horse (*Equus caballus*) and the donkey (*Equus asinus*). *Equine Veterinary Journal* **25** 14–18. (<https://doi.org/10.1111/j.2042-3306.1993.tb04816.x>)
- Parsons Aubone AM, Bisiau CM, McCue PM & Bouma GJ 2020 Presence of clock genes in equine full-term placenta. *Journal of Animal Science* **98** skaa094. (<https://doi.org/10.1093/jas/skaa094>)
- Posfai E, Rovic I & Jurisicova A 2019 The mammalian embryo's first agenda: making trophectoderm. *International Journal of Developmental Biology* **63** 157–170. (<https://doi.org/10.1387/ijdb.180404ep>)
- Raeside JJ, Christie HL, Waelchli RO & Betteridge KJ 2009 Estrogen metabolism by the equine embryo proper during the fourth week of pregnancy. *Reproduction* **138** 953–960. (<https://doi.org/10.1530/REP-09-0235>)
- Raeside JJ, Christie HL, Waelchli RO & Betteridge KJ 2012 Biosynthesis of oestrogen by the early equine embryo proper. *Reproduction, Fertility, and Development* **24** 1071–1078. (<https://doi.org/10.1071/RD11275>)
- Rambags BPB, van Tol HTA, van den Eng MM, Colenbrander B & Stout TAE 2008 Expression of progesterone and oestrogen receptors by early intrauterine equine conceptuses. *Theriogenology* **69** 366–375. (<https://doi.org/10.1016/j.theriogenology.2007.10.011>)
- Rapacz-Leonard A, Leonard M, Chmielewska-Krzyszewska M, Pazdzior-Czapula K & Janowski T 2018 Major histocompatibility complex class I in the horse (*Equus caballus*) placenta during pregnancy and parturition. *Placenta* **74** 36–46. (<https://doi.org/10.1016/j.placenta.2018.12.006>)
- Read JE, Cabrera-Sharp V, Offord V, Mirczuk SM, Allen SP, Fowkes RC & de Mestre AM 2018a Dynamic changes in gene expression and signalling during trophoblast development in the horse. *Reproduction* **156** 313–330. (<https://doi.org/10.1530/REP-18-0270>)
- Read JE, Cabrera-Sharp V, Kitscha P, Cartwright JE, King PJ, Fowkes RC & de Mestre AM 2018b Glial cells missing 1 regulates equine chorionic gonadotrophin beta subunit via binding to the proximal promoter. *Frontiers in Endocrinology* **9** 195. (<https://doi.org/10.3389/fendo.2018.00195>)
- Reinholt BM, Bradley JS, Jacobs RD, Ealy AD & Johnson SE 2017 Tissue organization alters gene expression in equine induced trophectoderm cells. *General and Comparative Endocrinology* **247** 174–182. (<https://doi.org/10.1016/j.ygcen.2017.01.030>)
- Riley P, Anson-Cartwright L & Cross JC 1998. The Hand1 bHLH transcription factor is essential for placental and cardiac morphogenesis. *Nature Genetics* **18** 271–275. (<https://doi.org/10.1038/ng0398-271>)
- Robles M, Peugnet P, Dubois C, Piumi F, Jouneau L, Bouchez O, Aubrière MC, Dahirel M, Aioun J, Wimel L *et al.* 2018 Placental function and structure at term is altered in broodmares fed with cereals from mid-gestation. *Placenta* **64** 44–52. (<https://doi.org/10.1016/j.placenta.2018.02.003>)
- Robles M, Loux S, de Mestre AM & Chavatte-Palmer P 2022 Environmental constraints and pathologies that modulate equine placental genes and development. *Reproduction* **163** R25–R38. (<https://doi.org/10.1530/REP-21-0116>)
- Saben J, Zhong Y, McKelvey S, Dajani NK, Andres A, Badger TM, Gomez-Acevedo H & Shankar K 2014 A comprehensive analysis of the human placenta transcriptome. *Placenta* **35** 125–131. (<https://doi.org/10.1016/j.placenta.2013.11.007>)
- Scaravaggi I, Borel N, Romer R, Imboden I, Ulbrich SE, Zeng S, Bollwein H & Bauersachs S 2019 Cell type-specific endometrial transcriptome changes during initial recognition of pregnancy in the mare. *Reproduction, Fertility, and Development* **31** 496–508. (<https://doi.org/10.1071/RD18144>)
- Seo MS, Park SB, Kim HS, Kang JG, Chae JS & Kang KS 2013 Isolation and characterization of equine amniotic membrane-derived mesenchymal stem cells. *Journal of Veterinary Science* **14** 151–159. (<https://doi.org/10.4142/jvs.2013.14.2.151>)
- Sferruzzi-Perri AN, Sandovici I, Constancia M & Fowden AL 2017 Placental phenotype and the insulin-like growth factors: resource allocation to fetal growth. *Journal of Physiology* **595** 5057–5093. (<https://doi.org/10.1113/jp273330>)
- Smits K, Govaere J, Peelman LJ, Goossens K, de Graaf DC, Vercauteren D, Vandaele L, Hoogewijs M, Wydooghe E, Stout T *et al.* 2012 Influence of

- the uterine environment on the development of in vitro-produced equine embryos. *Reproduction* **143** 173–181. (<https://doi.org/10.1530/REP-11-0217>)
- Soncin F, Khater M, To C, Pizzo D, Farah O, Wakeland A, Rajan KAN, Nelson KK, Chang CW, Moretto-Zita M *et al.* 2018 Comparative analysis of mouse and human placentae across gestation reveals species-specific regulators of placental development. *Development* **145**. (<https://doi.org/10.1242/dev.156273>)
- Stout TAE & Allen WR 2002 Prostaglandin E(2) and F(2 alpha) production by equine conceptuses and concentrations in conceptus fluids and uterine flushings recovered from early pregnant and dioestrous mares. *Reproduction* **123** 261–268. (<https://doi.org/10.1530/rep.0.1230261>)
- Wade CM, Giulotto E, Sigurdsson S, Zoli M, Gnerre S, Imsland F, Lear TL, Adelson DL, Bailey E, Bellone RR *et al.* 2009 Genome sequence, comparative analysis, and population genetics of the domestic horse. *Science* **326** 865–867. (<https://doi.org/10.1126/science.1178158>)
- Walters KW, Corbin CJ, Anderson GB, Roser JF & Conley AJ 2000 Tissue-specific localization of cytochrome P450 aromatase in the equine embryo by in situ hybridization and immunocytochemistry. *Biology of Reproduction* **62** 1141–1145. (<https://doi.org/10.1095/biolreprod62.5.1141>)
- Wang X, Miller DC, Clark AG & Antczak DF 2012 Random X inactivation in the mule and horse placenta. *Genome Research* **22** 1855–1863. (<https://doi.org/10.1101/gr.138487.112>)
- Wang X, Miller DC, Harman R, Antczak DF & Clark AG 2013 Paternally expressed genes predominate in the placenta. *PNAS* **110** 10705–10710. (<https://doi.org/10.1073/pnas.1308998110>)
- Yan Y, Peng H, Wang P, Wang H & Dong M 2016 Increased expression of fatty acid binding protein 4 in preeclamptic placenta and its relevance to preeclampsia. *Placenta* **39** 94–100. (<https://doi.org/10.1016/j.placenta.2016.01.014>)
- Yang QE, Giassetti MI & Ealy AD 2011 Fibroblast growth factors activate mitogen-activated protein kinase pathways to promote migration in ovine trophoblast cells. *Reproduction* **141** 707–714. (<https://doi.org/10.1530/REP-10-0541>)
- Zavy MT, Vernon MW, Sharp DC & Bazer FW 1984 Endocrine aspects of early pregnancy in pony mares: a comparison of uterine luminal and peripheral plasma levels of steroids during the estrous cycle and early pregnancy. *Endocrinology* **115** 214–219. (<https://doi.org/10.1210/endo-115-1-214>)
- Zhang YC, Qin XL, Ma XL, Mo HQ, Qin S, Zhang CX, Wei XW, Liu XQ, Zhang Y, Tian FJ *et al.* 2021 CLDN1 regulates trophoblast apoptosis and proliferation in preeclampsia. *Reproduction* **161** 623–632. (<https://doi.org/10.1530/REP-20-0677>)

Received 16 March 2021

First decision 27 April 2021

Revised manuscript received 6 January 2022

Accepted 13 January 2022