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Genomics, transcriptomics and proteomics of the normal and abnormal equine placenta: A better understanding of late gestational function and dysfunction

Genômica, transcriptômica e proteômica da placenta equina normal e anormal: um melhor entendimento da função final da gestação e disfunção

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Abstract

High throughput methods to assess genomics, gene (mRNA) expression, and protein composition of tissues and body fluids has led to a rapid advancement in understanding of normal and abnormal function of many body systems across a wide variety of mammalian and non-mammalian animal species. Over the past several years, our laboratory has applied these techniques to the study of normal physiology and disease of the pregnant mare, in particular for the placenta and fetal fluids. Although our understanding of the endocrine aspects of pregnancy in mares is reasonably advanced, much of our understanding related to placental function and dysfunction remains limited. This review will cover a number of studies which detail normal gestational changes in the fetal and maternal placenta along with changes in gene expression in a number of late gestational diseases of pregnancy.

Key words: *equine, placenta, transcriptome, placentitis, pregnancy*

Introduction

The advent of high-dimensional biological techniques including genomics, transcriptomics, proteomics, lipidomics and metabolomics as well as the increasing application of these techniques marks a landmark in research to understand normal physiologic function and the pathophysiology of disease in animals. In particular, high-throughput RNA sequencing allows quantitative evaluation of gene expression in a tissue or cell as well as investigation of different isoforms of transcript present. These approaches generate massive datasets which require high-capacity computing platforms for bioinformatics analysis. Fortunately, sequencing of the equine genome [1] and subsequent updates to the equine genome (EquCab3.0) [2] have provided detailed information about the equine genome. This information in combination with the explosion of information available regarding gene structure and function, as well as cellular, biological and disease pathways from biomedical research in humans and laboratory species has heralded a new era in veterinary medical research. The aim of this paper is to summarize recent studies in the authors' laboratory using these approaches to better understand normal physiology and disease of pregnancy in the mare.

Transcriptomics of the normal fetal and maternal placenta across gestation

Much of the research on the chorioallantois (CA) and endometrium (EN) as the fetal and maternal portions of the placenta in domestic animals has focused upon early pregnancy (maternal recognition of pregnancy) or very late preterm changes. We examined changes in messenger RNA (mRNA) transcripts in both the CA and EN across gestation in the mare including 1.5, 4, 6, 10 and 11 months of gestation [3]. A large number of differentially expressed genes (DGE) were identified with 5,932 and 3,667 DEG in CA and EN, respectively. The greatest difference in expression was seen at 4 or 11 month GA. Unsurprisingly, many of the most highly expressed genes in CA and EN related to either endocrine or immune function. Highly expressed genes include endocrine-related transcripts (*RLN, CYP19A1, HSD3B2, SPP1, PLA2G10, INHBA*), immune-related transcripts (*CST3, CTSL, SERPINA3, SERPINA6, SERPINA14, SPINK7, SPINK9, LTF, S100A6, SLPI*), iron-binding proteins (*ACP5, FTH1, HBA2, LCN2, SERPINA14*), and serine protease inhibitors (*SERPINA3, SERPINA6, SERPINA14, SPINK7, SPINK9*). Others include extracellular matrix proteins (*ECM1, SPARC, MMP26*), transport proteins (*ACP5, GM2A, HBA2, LCN2*), and antioxidants (*PRDX1, SOD3*). Evaluation of gene expression networks provides the ability to examine cellular or biological pathways associated with changes in

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transcript abundance. In the fetal and maternal placenta, many pathways associated with cell growth, mitosis, metabolism, oxidative stress, angiogenesis and steroidogenesis were upregulated while immune related pathways including B-cell activation, leukocyte and lymphocyte activation, immune response were downregulated. These findings are consistent with the needs of the fetal placenta for continued growth, steroid synthesis and transport of materials to the fetus along with protecting the allogeneic fetus from the maternal immune response.

Analysis of the transcriptome of the placenta across gestation has also been useful in better understanding the endocrine function of both the EN and CA during equine pregnancy [4]. In particular, evaluation of various isoforms of steroidogenic enzymes in the CA and EN across gestation reveals a close coordination between these two tissues for the regulation of pregnane and estrogen synthesis during pregnancy. Likewise, changes in steroid receptors during gestation imply differences in relative importance of receptor types during pregnancy. The nuclear progesterone receptor has a low, relatively constant expression in CA and EN for most of gestation; however, membrane-associated progesterone receptor (*PGRMC1*) is highly expressed in EN and CA which suggest that placental effects of pregnanes in mares may be mediated by these receptors in mares [4].

MicroRNA in chorioallantois, endometrium and circulation in normal and abnormal equine pregnancy

In addition to protein-coding messenger RNA (mRNA), there are a variety of small non-coding RNA (sncRNA) that are detected with RNA-sequencing. These sncRNA appear to regulate the expression of mRNA and provide additional information about gene function. Of the sncRNA, microRNA (miRNA) are small (20-22 nucleotides) RNA that regulate protein-coding genes. Expression of miRNA clusters have been described in the human placenta including one on human chromosome 14 (C14MC) which appears to be highly conserved across eutherian mammals and seems to play an important role in placental development [5]. In the horse, the orthologous miRNA cluster is located on ECA24 (C24MC) [6]. The expression of miRNA in CMC24 in the equine placenta was higher in earlier pregnancy and declined with advancing gestation [6]. Target mRNA of the miRNA in the C24MC cluster had a reciprocal expression pattern (increased with gestational age), and many of the transcripts regulated by these miRNAs were related to angiogenesis and vascularization of the placenta with advancing pregnancy [6].

Unlike mRNA, miRNA have a relatively long half-life in circulation, and changes in circulating miRNA have been identified in normal and abnormal pregnancy in animals and in humans with potential application as diagnostic biomarkers [7-10]. In an initial, PCR-based study, we identified one miRNA that was differentially expressed in late gestation (miR-374b) and four miRNA that were differentially expressed during pregnancy in mares (miR-454, miR-133b, miR-486-5p and miR-204b) [11]. These pregnancy-specific miRNA targeted pathways related to placentation, angiogenesis and endocrine function during pregnancy [11]. Members of the C24MC cluster of miRNA also were detected in circulation of pregnant mares [12]. MicroRNA from the C24MC cluster were more highly expressed in circulation during early pregnancy, consistent with their expression pattern in the placenta. The serum enrichment with miR-1247-3p, miR-134-5p, miR-382-5p, and miR-433-3p at 25 d pregnancy as well as miR-1247-3p, miR-134-5p, miR-409-3p, and miR-379-5p at 45 d pregnancy suggest that these miRNAs are involved in early pregnancy events [12].

One of the goals of examining miRNA expression in serum during pregnancy was to evaluate the use of miRNA in blood as potential biomarkers during abnormal equine pregnancy. For this study, RNA-Seq was used to screen ncRNA expression in the CA, EN and blood of mares with experimentally induced placentitis at approximately 280 days of gestation [13]. For this study, tissues were collected between 3 to 5 days after inoculation in treated mares, and uninoculated mares of comparable gestational age were used as controls [13]. Analysis of ncRNA expression in blood, CA and EN revealed 658 and 507 miRNA for tissue and blood respectively. Principal component analysis of these data revealed distinct clustering of samples based upon tissue of origin and disease state. A total of 50 ncRNA were differentially expressed between control and placentitis tissue samples. Differentially expressed miRNA included 26 in CA, 20 in EN and 9 in serum. Of nine miRNA which were changed in serum, six also exhibited parallel changes in either CA or EN [13]. Many of the miRNA which were upregulated in equine placentitis were also upregulated in studies in women with chorioamnionitis suggesting that aspects of these disease processes are conserved. Many of the miRNA which were dysregulated in equine placentitis

were associated with altered immune function. In particular, regulation of the inflammatory-mediating cytokine, IL-6 and IL-8, as well as activation of macrophage and lymphocytes. Whether or not the changes identified in circulating miRNA in mares with experimental placentitis will have utility in diagnosis of spontaneous equine placentitis remains to be seen. Although changes detected were statistically significant between controls and treated mares, the magnitude of changes may not lend themselves to good diagnostic tests, and it is likely that a panel of miRNA will need to be evaluated as possible biomarkers.

Changes in the transcriptome during abnormal equine pregnancy

Nocardioform placentitis

Nocardioform placentitis remains a poorly understood disease of the placenta in mares. It is characterized by late-term abortions and fetal growth retardation associated with a distinct placental lesion located typically at the ventral aspect of the placenta, distinct from the cervical star. Nocardioform placentitis is associated with gram-positive, branching actinomycetes including *Amycolatopsis* spp., and *Crossiela equi* along with more recently characterized isolates of *Streptomyces atiruber* and *Streptomyces silaceus* [14-16]. During the 2017 foaling season, we collected placenta from mares with suspect nocardioform placentitis (n = 4) and four normal placentae as controls. RNA isolated from these tissues was analyzed by RNA-Seq [17]. A total of over 3,000 genes were differentially expressed in placenta from mares with NP. Signaling pathways related to inflammation (cytokines and chemokines), pattern recognition receptors (toll-like receptors), apoptosis (caspases), hypoxia, angiogenesis and antimicrobial peptides were upregulated in placenta from mares with nocardioform placentitis compared to normal term placenta.

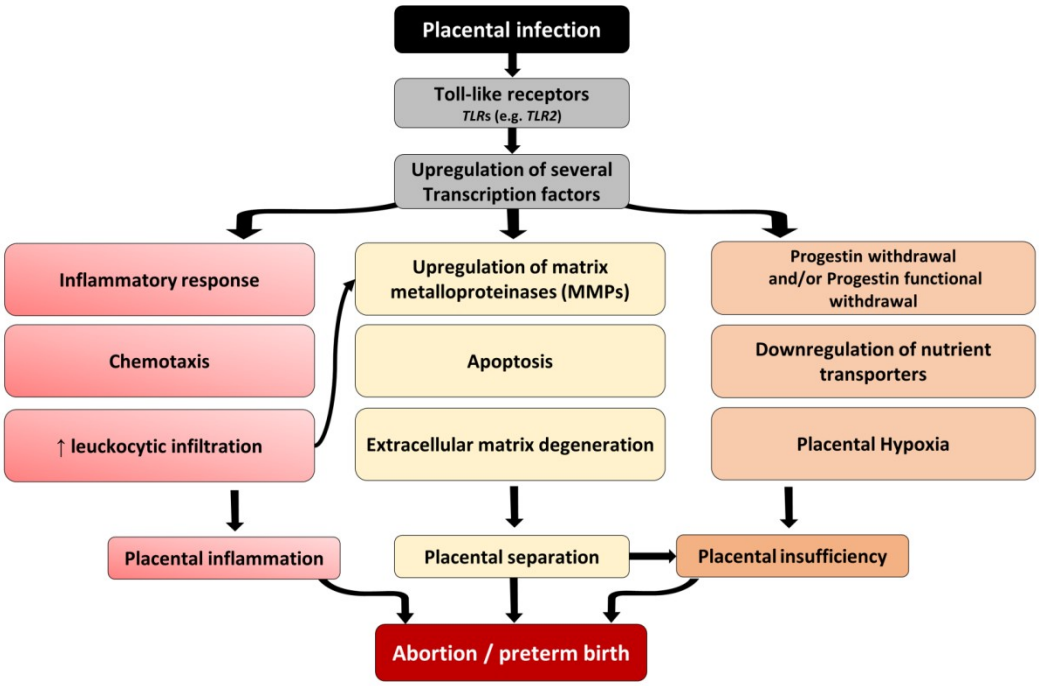

Figure 1. Schematic illustration of possible mechanisms associated with ascending placentitis in the mare.

Ascending bacterial placentitis

In addition to nocardioform placentitis, ascending bacterial placentitis remains an important cause of late term pregnancy loss in mares. We examined changes in the transcriptome of both the CA and EN recovered from mares with experimentally induced placentitis (*Streptococcus equi* spp. *zooepidemicus*) at eight days after initial transcervical inoculation at around 290 d GA. Again,

uninoculated mares served as controls. Again, almost 3000 genes were differentially expressed in CA and almost 1000 DEG were detected in EN from mares with ascending placentitis. Upregulated pathways in CA included inflammation, interleukin and integrin signaling, angiogenesis, apoptosis, toll-receptor signaling as well as B-cell and T-cell activation (El-Sheikh Ali and Ball, unpublished). Upregulated pathways in EN included inflammation and integrin signaling, as well as toll receptor signaling. These changes were in turn associated with dysregulation of placental steroidogenesis, angiogenesis, nutrient transport as well as hypoxia. A number of matrix metalloproteases were also upregulated in CA which may be associated with degradation of the extra-cellular matrix and resultant placental separation. Related pathways and mechanisms associated with this dataset are illustrated (Figure 1).

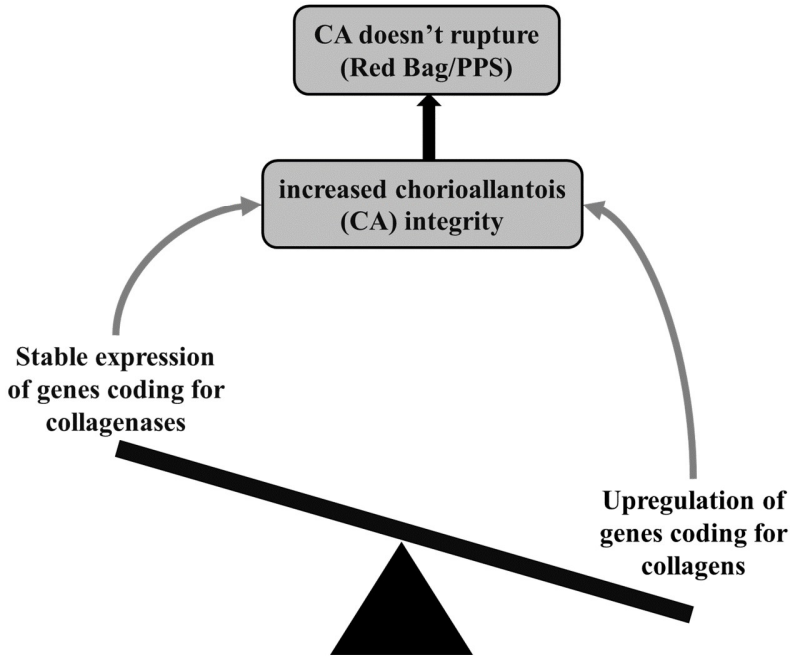

Figure 2. Schematic diagram of changes associated with premature placental separation in the mare.

Premature placental separation

Premature placental separation (red bag placenta; PPS) is a common and poorly understood problem in the foaling mare. Premature separation of the chorioallantois from the endometrium without rupture of the chorioallantois at the cervical star during late first stage and second stage labor may result in significant fetal hypoxia if not identified quickly and corrected by opening the chorioallantois to assist delivery of the foal. The incidence of PPS varies with study, but was cited as 1.6% of 1,047 foalings with a mortality rate of 17.6% [18], 0.9% of abortions presented in Normandy France [19] or 4.7% of reproductive losses in central Kentucky [20]. During late abortions associated with Mare Reproductive Loss Syndrome (MRLS), the incidence of PPS was reported as 28% of cases [21]. These data likely underestimate the frequency of PPS because many such cases are likely not presented to diagnostic laboratories if the neonate is not overtly affected during delivery. Clinically, PPS has been variably associated with problems such as endophyte-infected fescue [22], placental inflammation associated with viral (EHV-1) placentitis [23] and ascending bacterial placentitis [24]. Although clinical presentation of equine PPS is well known, the underlying pathophysiology of the problem in the mare is poorly understood. Therefore, we examined holistic changes in gene expression in the chorioallantois of mares with premature placental separation using next-generation sequencing technology. In this study, we performed RNA-Seq on CA from mares with PPS (n = 33) and mares with normal parturition as controls (n = 4). Resulting RNA-Seq data were subjected to a standard analysis pipeline to examine differentially expressed genes and

associated pathways and upstream regulators (Murase, El Sheikh Ali and Ball, unpublished data). Again, a large number of DEGs was detected between control CA and CA from mares with PPS. A number of genes associated with extracellular matrix, including collagens, proteoglycans, and metalloproteinase inhibitors were upregulated in mares with PPS compared to control CA. Key upstream regulators included transcripts associated with hypoxia, inflammation, extracellular matrix and cell adhesion (Figure 2).

Hydrops allantois

Hydrops conditions are rare in the mare and there is little information about the underlying pathogenesis of these diseases in any species. We evaluated the CA from formalin-fixed paraffin embedded (FFPE) tissues collected from archival materials of cases of hydrops allantois submitted to the

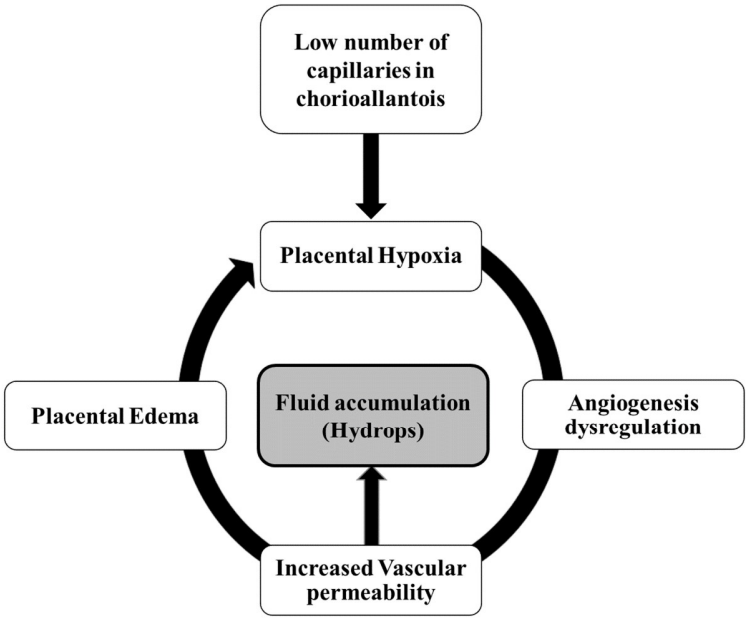

Figure3. Schematic illustration of changes associated with hydrops allantois in the mare.

UKVDL (n = 10) compared to FFPE of gestationally age-matched normal control mares. RNA was isolated from FFPE tissues from both groups for assessment of expression of genes related to angiogenesis and steroidogenesis [25]. Capillary density was reduced and expression of angiogenic genes was lower in CA from hydrops allantois cases while transcripts related to hypoxia were increased compared to controls (Figure 3) [26]. Interestingly, expression of genes associated with estrogen synthesis and estrogen receptors were also downregulated, which suggests a possible role of estrogen in dysregulation of placental angiogenesis in these cases.

Proteomic analysis of equine fetal fluids in normal and abnormal pregnancy

Analysis of biological materials by mass spectrometry can also be used to determine the protein composition of body fluids, including amniotic and allantoic fluids [27, 28]. The proteome of fetal fluids from control mares and from mares with experimentally induced placentitis was determined by LTQ-Orbitrap mass-spectrometry [27, 28]. Overall, a total of 130 proteins were characterized in amniotic and/or allantoic fluid with a total of 18 proteins upregulated in amniotic fluid from mares with placentitis. Three

proteins were present only in amniotic fluid in placentitis (haptoglobin, plasminogen isoforms). An additional 15 proteins were upregulated in amniotic fluid including proteins in the serpin superfamily, immunoglobulins, apolipoproteins, transferrin, thyroxine-binding globulin and serum albumin [28]. Interestingly, positive acute-phase proteins (haptoglobin, alpha-1antiproteinase, and alpha-2-macroglobulin and negative acute phase proteins (transferrin albumin) both increased in amniotic fluid from mares with inflamed placenta. A number of these proteins are known to regulated by the inflammatory modulating cytokine, IL-6 and have been shown to change during placental inflammation in women as well [28]. Allantoic fluid had relatively fewer proteins change in the presence of placentitis and most of these proteins were in common with those of amniotic fluid (alpha-1-antiproteinase, serotransferrin and transferrin). Similar results were obtained in a second study which found that transferrin, lactoferrin and alpha-1-antiproteinase were increased in allantoic fluid of mares with placentitis [27]. Alpha-1-antiproteinase is an anti-inflammatory protein which acts to modulate tissue damaging effects of neutrophil enzymatic proteins and serum concentrations of this protein have been used as an acute-phases protein for detection of inflammation in humans [27].

Conclusions

This review provides a brief overview of the application of high-dimensional biology to the study of normal and abnormal equine pregnancy. Data for these studies is (or will be) available in public depositories and hopefully will provide researchers with a valuable resource in coming years both to address specific research questions as well as formulate new research hypotheses concerning pregnancy in the mare.

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