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

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Abstract

High-throughput methods to assess genomics, gene messenger RNA expression, and protein composition of tissues and body fluids have led to rapid advancement in understanding of normal and abnormal function of many body systems across a wide variety of animal (mammalian and nonmammalian) species. Over several years, these techniques were applied to study normal physiology and disease of pregnant mare, focusing on placental and fetal fluids. Although our understanding of endocrine aspects of pregnancy in mares is reasonably advanced, much of our understanding related to placental function and dysfunction remains limited. This review covers studies that detailed normal pregnancy changes in fetal and maternal placenta, along with changes in gene expression in a number of late-pregnancy diseases.

Keywords: Equine, placenta, transcriptome, placentitis, pregnancy

Introduction

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

Transcriptomics of normal fetal and maternal placenta across pregnancy

Much research on chorioallantois (CA) and endometrium (EN) as fetal and maternal portions of placenta in domestic animals has focused on early pregnancy (maternal recognition of pregnancy) or very late pre-term changes. We examined changes in messenger RNA (mRNA) transcripts in both CA and EN across pregnancy (1.5, 4, 6, 10, and 11 months) in mare.[3] Large number of differentially expressed genes (DGE) were identified, with 5,932 and 3,667 DEG in CA and EN, respectively. Greatest difference in expression occurred at 4 or 11 months of pregnancy. Unsurprisingly, most highly expressed genes in CA and EN were related to either endocrine or immune function. Highly expressed genes included 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 included extracellular matrix proteins (ECM1, SPARC, MMP26), transport proteins (ACP5, GM2A, HBA2, LCN2), and antioxidants (PRDX1, SOD3). Evaluation of gene expression networks provides ability to examine cellular or biological pathways associated with changes in transcript abundance. In fetal and maternal placenta, many pathways associated with cell growth, mitosis, metabolism, oxidative stress, angiogenesis, and steroidogenesis were upregulated, whereas immune-related pathways, including B cell activation, leukocyte and lymphocyte activation and immune response, were downregulated. These findings are consistent with fetal placenta

needs for continued growth, steroid synthesis, and transport of materials to fetus, along with protecting allogeneic fetus from maternal immune response.

Analysis of placental transcriptome across pregnancy has also been useful in better understanding endocrine function of both EN and CA during equine pregnancy.[4] In particular, evaluation of various isoforms of steroidogenic enzymes in CA and EN across pregnancy reveals close coordination between these 2 tissues for pregnane and estrogen synthesis regulation during pregnancy. Similarly, changes in steroid receptors during pregnancy imply differences in relative importance of receptor types during pregnancy. Nuclear progesterone receptor has lower and relatively constant expression in CA and EN for most of pregnancy; 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 noncoding RNA (ncRNA) that are detected with RNA-Seq. These ncRNA appear to regulate expression of mRNA and provide additional information about gene function. Of ncRNA, microRNA (miRNA) are small (20 - 22 nucleotides) RNA that regulate protein coding genes. Expression of miRNA clusters have been described in human placenta, including human chromosome 14 (C14MC) that appears to be highly conserved across eutherian mammals and seems to have an important role in placental development.[5] In horse, orthologous miRNA cluster is located on ECA24 (C24MC).[6] Expression of miRNA in C24MC in equine placenta was higher in earlier pregnancy, but declined with advancing pregnancy.[6] Target mRNA of miRNA in C24MC cluster had a reciprocal expression pattern (increased with pregnancy), and many transcripts regulated by these miRNAs were related to angiogenesis and vascularization of 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 1 miRNA that was differentially expressed in late pregnancy (miR-374b) and 4 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 C24MC cluster of miRNA were also detected in circulation of pregnant mares.[12] MicroRNA from C24MC cluster were more highly expressed in circulation during early pregnancy, consistent with their expression pattern in placenta. Serum enrichment with miR-1247-3p, miR-134-5p, miR-382-5p, and miR-433-3p at day 25 pregnancy and miR-1247-3p, miR-134-5p, miR-409-3p, and miR-379-5p at day 45 pregnancy suggest that these miRNAs are involved in early pregnancy events.[12]

One goal of examining miRNA expression in serum during pregnancy was to evaluate use of miRNA in blood as potential biomarkers during abnormal equine pregnancy. For this study, RNA-Seq was used to screen ncRNA expression in CA, EN and blood of mares with experimentally induced placentitis at ~ 280 days of pregnancy.[13] Tissues collected between 3 - 5 days after inoculation in treated mares and uninoculated mares of comparable pregnancy 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 9 miRNA that changed in serum, 6 also exhibited parallel changes in either CA or EN.[13] Many miRNA that were upregulated in equine placentitis were also upregulated in women with chorioamnionitis, suggesting that aspects of these disease processes are conserved. Many miRNA that were dysregulated in equine placentitis were associated with altered immune function, in particular, regulation of inflammation mediating cytokines IL6 and IL8, as well as activation of macrophages and

lymphocytes. Whether or not changes identified in circulating miRNA in mares with experimental placentitis will have utility in diagnosis of spontaneous equine placentitis remains to be determined. Although different changes detected between controls and treated mares, 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 transcriptome during abnormal equine pregnancy

Nocardioform placentitis

Nocardioform placentitis (NP) remains a poorly understood disease of placenta in mares. It is characterized by late-term abortions and fetal growth retardation associated with a distinct placental lesion located typically at ventral aspect of placenta, distinct from cervical star. NP is associated with gram-positive, branching actinomycetes including *Amycolatopsis* spp., *Crossiela equi* along with more recently characterized isolates of *Streptomyces atiruber* and *Streptomyces silaceus*.[14-16] During 2017 foaling season, we collected placenta from mares suspect for nocardioform placentitis (n = 4) and 4 normal placentas 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 upregulated in placenta from mares with NP were compared to normal term placenta.

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 transcriptome of both CA and EN recovered from mares with experimentally induced placentitis (*Streptococcus equi* spp. *zooepidemicus*) at 8 days after initial transcervical inoculation and ~ 290 days of pregnancy. Uninoculated mares served as controls; ~ 3000 genes were differentially expressed in CA and ~ 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, and B cell and T cell activation (El-Sheikh and Ball, unpublished). Upregulated pathways in EN included inflammation and integrin signaling, and toll receptor signaling. These changes, in turn, were associated with dysregulation of placental steroidogenesis, angiogenesis, nutrient transport, and hypoxia. A number of matrix metalloproteases were also upregulated in CA that may be associated with degradation of extracellular matrix and resultant placental separation. Related pathways and mechanisms associated with this dataset are illustrated (Figure 1).

Premature placental separation

Premature placental separation (PPS; red bag placenta) is a common and poorly understood problem in foaling mare. Premature separation of CA from endometrium without rupture of CA at cervical star during late first stage and second stage labor may result in significant fetal hypoxia if not identified quickly and corrected by opening chorioallantois to assist foal delivery. Incidence of PPS varies with study, but cited as 1.6% of 1,047 foaling 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), incidence of PPS was reported as 28% of cases.[21] These data likely underestimate PPS frequency, because many such cases are likely not presented to diagnostic laboratories if 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, underlying pathophysiology of problem in mare is poorly understood. Therefore, we examined holistic changes in gene expression in CA of mares with premature placental separation using next generation sequencing technology. We performed RNA-Seq on CA from

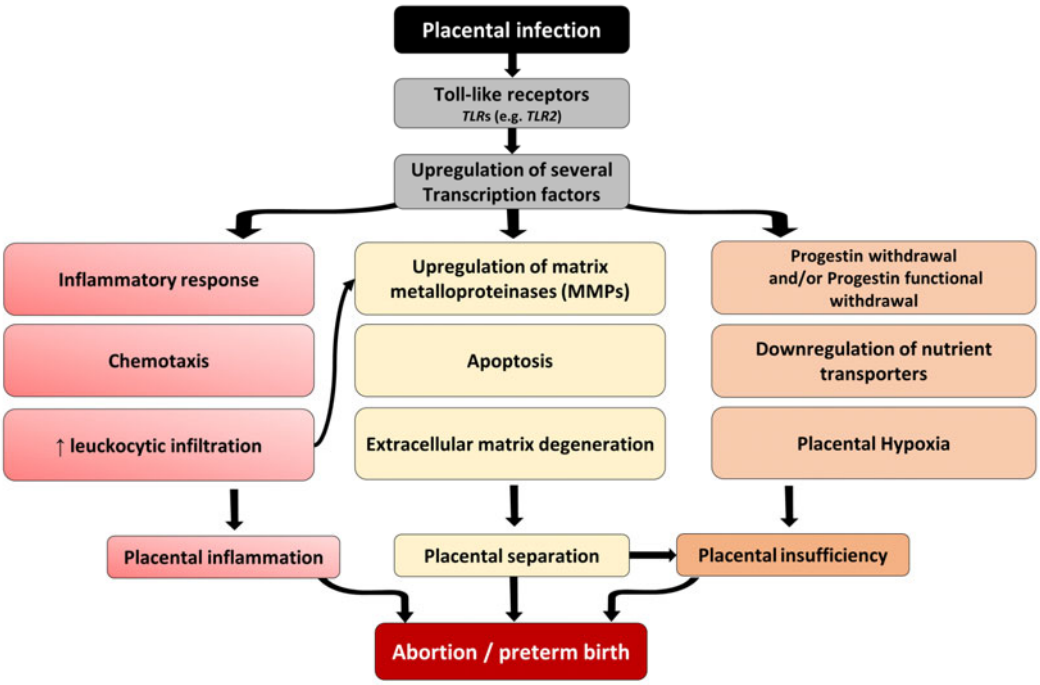

Figure 1. Schematic illustration of possible mechanisms associated with ascending placentitis in mare.

mares with PPS (n = 33) and mares with normal parturition as controls (n = 4). Resulting RNA-Seq data were subjected to standard analysis pipeline to examine differentially expressed genes and associated pathways and upstream regulators (Murase, El Sheikh Ali and Ball, unpublished data). A large number of differentially expressed genes (DGE) were identified with 5,932 and 3,667 DEG in CA and EN, respectively. 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 identified include transcripts associated with hypoxia, inflammation, extracellular matrix, and cell adhesion (Figure 2).

Hydrops allantois

Hydrops conditions are rare in mare and there is little information about underlying pathogenesis of these diseases in any species. We evaluated CA from formalin-fixed paraffin embedded (FFPE) tissues collected from archival materials of cases of hydrops allantois submitted to University of Kentucky Veterinary Diagnostic Laboratory (n = 10) compared to FFPE of pregnancy-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 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.

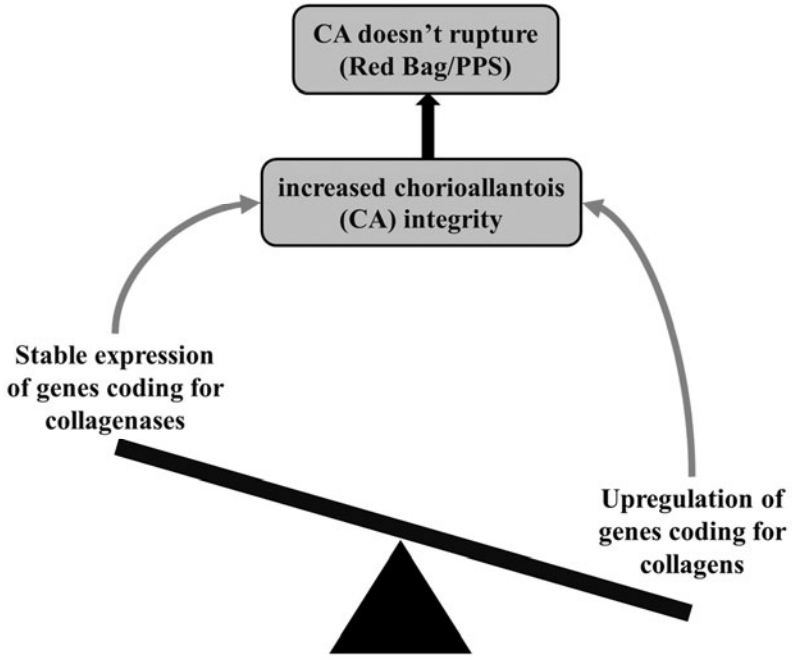

Figure 2. Schematic diagram of changes associated with premature placental separation in mare.

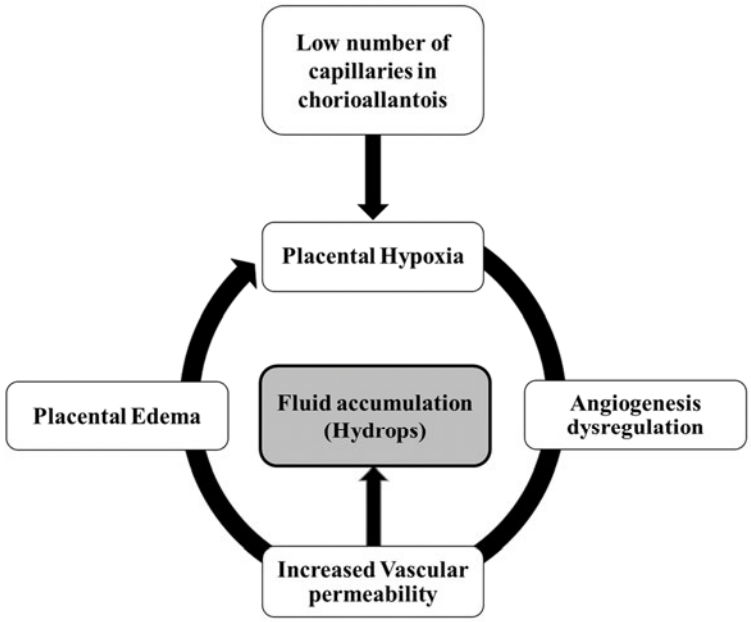

Figure 3. Schematic illustration of changes associated with hydrops allantois in mare.

Proteomic analysis of equine fetal fluids in normal and abnormal pregnancy

Analysis of biological materials by mass spectrometry can also be used to determine protein composition of body fluids, including amniotic and allantoic fluids.[27,28] 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 (haptoglobin, plasminogen isoforms) were present only in amniotic fluid in placentitis. An additional 15 proteins were upregulated in amniotic fluid, including proteins in serpin superfamily, immunoglobulins, apolipoproteins, transferrin, thyroxine binding globulin and serum albumin.[28] Interestingly, both, positive acute phase proteins (haptoglobin, alpha-1antiproteinase, and alpha-2-macroglobulin) and negative acute phase proteins (transferrin albumin) were increased in amniotic fluid from mares with inflamed placenta. A number of these proteins are regulated by inflammatory modulating cytokine (IL6) and change during placental inflammation in women.[28] Allantoic fluid had relatively fewer proteins change in placentitis presence 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 (increases in transferrin, lactoferrin and alpha-1-antiproteinase in allantoic fluid of mares with placentitis).[27] Alpha-1-antiproteinase is an anti-inflammatory protein and modulates tissue-damaging effects of neutrophil enzymatic proteins. Serum concentrations of this protein are used as an acute-phase protein to detect inflammation in humans.[27]

Conclusion

Application of high-dimensional biology to normal and abnormal equine pregnancy is essential. Data from our studies provide researchers a valuable resource to address specific research questions and to formulate new research hypotheses concerning pregnancy in mare.

Acknowledgement

Supported by Albert G. Clay Endowment of University of Kentucky.

Conflict of interest

None to report.

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