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Repository Citation

Loux, Shavahn C.; Scoggin, Kirsten E.; Bruemmer, Jason E.; Canisso, Igor F.; Troedsson, Mats H. T.; Squires, Edward L.; and Ball, Barry A., "Evaluation of Circulating miRNAs During Late Pregnancy in the Mare" (2017). *Veterinary Science Faculty Publications*. 35.
https://uknowledge.uky.edu/gluck_facpub/35

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Evaluation of Circulating miRNAs During Late Pregnancy in the Mare**Notes/Citation Information**

Published in *PLOS ONE*, v. 12, 4, e0175045, p. 1-12.

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Digital Object Identifier (DOI)

<https://doi.org/10.1371/journal.pone.0175045>

RESEARCH ARTICLE

Evaluation of circulating miRNAs during late pregnancy in the mare

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OPEN ACCESS

Citation: Loux SC, Scoggin KE, Bruemmer JE, Canisso IF, Troedsson MHT, Squires EL, et al. (2017) Evaluation of circulating miRNAs during late pregnancy in the mare. PLoS ONE 12(4): e0175045. <https://doi.org/10.1371/journal.pone.0175045>

Editor: Peter J. Hansen, University of Florida, UNITED STATES

Received: December 13, 2016

Accepted: March 20, 2017

Published: April 7, 2017

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was funded by the Kentucky Thoroughbred Association/Kentucky Thoroughbred Breeders and Owners (<http://www.kentuckybred.org>), the Albert Clay Endowment and the Paul Mellon post-doctoral fellowship, and the Geoffrey Hughes doctoral fellowship. Due to their relatively small size, not all funding sources have a website or grant numbers. The funders had no role

Abstract

MicroRNAs (miRNAs) are small, non-coding RNAs which are produced throughout the body. Individual tissues tend to have a specific expression profile and excrete many of these miRNAs into circulation. These circulating miRNAs may be diagnostically valuable biomarkers for assessing the presence of disease while minimizing invasive testing. In women, numerous circulating miRNAs have been identified which change significantly during pregnancy-related complications (e.g. chorioamnionitis, eclampsia, recurrent pregnancy loss); however, no prior work has been done in this area in the horse. To identify pregnancy-specific miRNAs, we collected serial whole blood samples in pregnant mares at 8, 9, 10 m of gestation and post-partum, as well as from non-pregnant (diestrous) mares. In total, we evaluated a panel of 178 miRNAs using qPCR, eventually identifying five miRNAs of interest. One miRNA (miR-374b) was differentially regulated through late gestation and four miRNAs (miR-454, miR-133b, miR-486-5p and miR-204b) were differentially regulated between the pregnant and non-pregnant samples. We were able to identify putative targets for the differentially regulated miRNAs using two separate target prediction programs, miRDB and Ingenuity Pathway Analysis. The targets for the miRNAs differentially regulated during pregnancy were predicted to be involved in signaling pathways such as the STAT3 pathway and PI3/AKT signaling pathway, as well as more endocrine-based pathways, including the GnRH, prolactin and insulin signaling pathways. In summary, this study provides novel information about the changes occurring in circulating miRNAs during normal pregnancy, as well as attempting to predict the biological effects induced by these miRNAs.

Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs whose expression varies by tissue, with individual tissues exhibiting unique miRNA fingerprints [1]. The primary biological function of miRNAs is to repress translation of messenger RNAs (mRNA) by binding to highly complementary targets, typically on the 3' untranslated region. Degradation of the target mRNA may

in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: One of our funding sources was a commercial source: the Kentucky Thoroughbred Association/Kentucky Thoroughbred Breeders and Owners. Although this is a commercial association, there are no ties to them through employment, consultancy, patents, products in development or marketed products. This group is comprised of local horse owners and breeders who are interested in learning more about the biology of pregnancy in the hopes of eventually reducing the incidence of late-term abortions in their own stock. Funding from this source does not alter our adherence to PLOS ONE policies on sharing data and materials.

or may not occur, depending on the degree of complementarity [1]. Many of these miRNAs are released into circulation, where they are bound to carrier proteins or lipoproteins such as high-density lipoprotein, or, more commonly, contained within membrane-bound vesicles known as exosomes [2, 3]. Their presence and longevity in circulation has made them attractive targets as biomarkers for a variety of conditions, including pregnancy disorders [4].

The recent interest surrounding miRNAs as biomarkers is well founded for a number of reasons. First, they are exceptionally stable within circulation with an estimated half-life of more than five days [5]. Additionally, their expression changes significantly throughout the course of many diseases and physiological conditions, including cancer [3, 6], cardiovascular disease [7], metabolic diseases (e.g. diabetes) [8], skeletal diseases [9], and pregnancy disorders [10]. Pregnancy-associated diseases are of particular interest due to the difficulties in diagnosing many of these diseases in the early stages combined with their potentially catastrophic consequences in any species. Earlier diagnosis allows for earlier intervention and a more favorable outcome.

In humans, circulating miRNA levels have been shown to change significantly during pre-eclampsia, ectopic pregnancy, intra-uterine growth restriction and pre-term labor [11, 12]. There has been no information about the expression of circulating miRNAs in late-term equine pregnancy; however, a recent study identified twelve exosomal circulating miRNAs proposed to be involved with maternal recognition of pregnancy [13]. Circulating miRNAs have tremendous diagnostic potential for many similar pregnancy-related complications affecting horses and humans, including fetal growth restriction and placental infection. Understanding the physiological changes in miRNA expression during normal gestation gives us important information about the role of miRNAs during pregnancy.

To the best of our knowledge, there has been no previous research on the normal expression of circulating miRNAs in whole blood from mares during late gestation. Therefore, we have evaluated miRNA expression profiles throughout late gestation and compared them to non-pregnant mares, as well as evaluating physiological changes through late gestation.

Materials and methods

All animal procedures were prospectively approved by and completed in accordance with the Institutional Animal Care and Use Committee of the University of Kentucky (Protocol# 2010–0769). All horses used in this study were light-breed mares owned by the University of Kentucky, acquired through the breeding program or by donation. Mares ranged from 350–550 kg and were between 7 and 16 years of age. Mares were housed on pasture with free-choice grass hay available at all times. All horses were retained following the completion of this study. Gestational age was determined using the day of ovulation (Day 0).

Experimental design

Whole blood was collected using PAXgene blood collection tubes (PreAnalytiX; Hombrechtikon, Switzerland) serially from mares at 8, 9, 10 m gestation & post-partum ($n = 5$) as well as in mares at 9 d diestrus ($n = 4$) to compare circulating miRNAs between pregnant and non-pregnant mares, as well as to determine stability of miRNA expression through late gestation. Post-partum samples were taken 14–31 days post-foaling. PAXgene tubes were immediately frozen at -20°C . Real-time PCR was used to evaluate the relative expression of 178 mature equine miRNAs within these samples (S1 Table). All mares were confirmed to have normal pregnancies by ultrasonography at the sampling times and thorough pathologic placental evaluation postpartum.

RNA purification/qPCR

RNA was purified using the PAXgene blood miRNA kit (Qiagen, Germantown, MD, USA) per manufacturer's instructions, then stored at -20°C until use. RNA purity and concentration were assessed using the NanoDrop 2000 spectrophotometer (ThermoScientific, Waltham, MA, USA), with 260/280 ratios above 1.8 and 260/230 ratios above 2.0 being considered acceptable.

Libraries of cDNA were created using the miScript Reverse Transcription kit (Qiagen) with the HiSpec® buffer to select for mature miRNAs. A total of 400 ng RNA was used per reaction, following manufacturer's instructions. In short, 4 μL 5x HiSpec buffer, 2 μL 10x miScript Nucleics Mix, 2 μL miScript Reverse Transcriptase Mix were used, with nuclease-free water added to bring the final volume to 20 μL . An additional aliquot was made with water in place of the RNA for a negative control. Tubes were held at 37°C for 60 min followed by 5 min at 95°C , then cooled to 4°C where it was held until use.

Reactions were immediately prepared for qPCR using the miScript SYBR Green PCR kit (Qiagen). In short, each reaction consisted of 3 μL 2x QuantiTect SYBR Green PCR Master Mix, 0.6 μL 10x miScript Universal Primer, 1.34 μL nuclease-free H_2O , 1.5 μL miRNA-specific forward primer and 0.06 μL cDNA. All reactions were performed in duplicate. The RNA-free reverse transcription reaction and a cDNA-free PCR reaction were used as negative controls. Thermal cycling took place on a LightCycler 480 PCR system (Roche, Indianapolis, IN, USA), and consisted of an initial incubation at 95°C for 15 min followed by 40 cycles at 95°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. Melt curve analysis was performed to confirm amplification of a single cDNA product.

Data were normalized by $\Delta\text{Ct} = \text{Ct}_{(\text{target})} - \text{Ct}_{(\text{mean of sample})}$. This method of normalization has been shown to be superior to identifying select housekeeping genes for large-scale miRNA expression studies [14].

MicroRNA target evaluation

Predicted targets of differentially expressed miRNAs were assessed using miRDB [15], with mRNAs with a target prediction score (TPS) of ≥ 80 selected for further analysis as suggested by the manufacturer. The predicted mRNA targets for each miRNA are listed in [S2 File](#).

To assess which pathways were affected by the altered miRNA expression, predicted target mRNAs were analyzed using Ingenuity Pathway Analysis (Qiagen, Redwood City, CA, USA). The mRNAs were grouped based on the status of the targeting miRNA, and a core analysis was run on each group. Canonical pathways were derived from each core analysis.

Statistics

Data analyses were performed using JMP 11 (SAS Institute, Cary, NC, USA). Variability of miRNA expression through late gestation was analyzed using one-way ANOVA adjusted for false-discovery rate with the Benjamini-Hochberg adjustment ($P < 0.1$), with the ΔCt value as the Y variable and the stage of gestation, or pregnancy status as the X variable [16]. Post-hoc analysis was performed using student's t-test for pregnant vs diestrus ($P < 0.05$).

Results

Late gestation/diestrus analysis

Only a single miRNA derived from whole blood was differentially regulated through the late stages of gestation, miR-374b. Post-hoc analysis confirmed expression of miR-374b was significantly increased at all stages (8, 9 and 10 m) of gestation when compared to post-partum

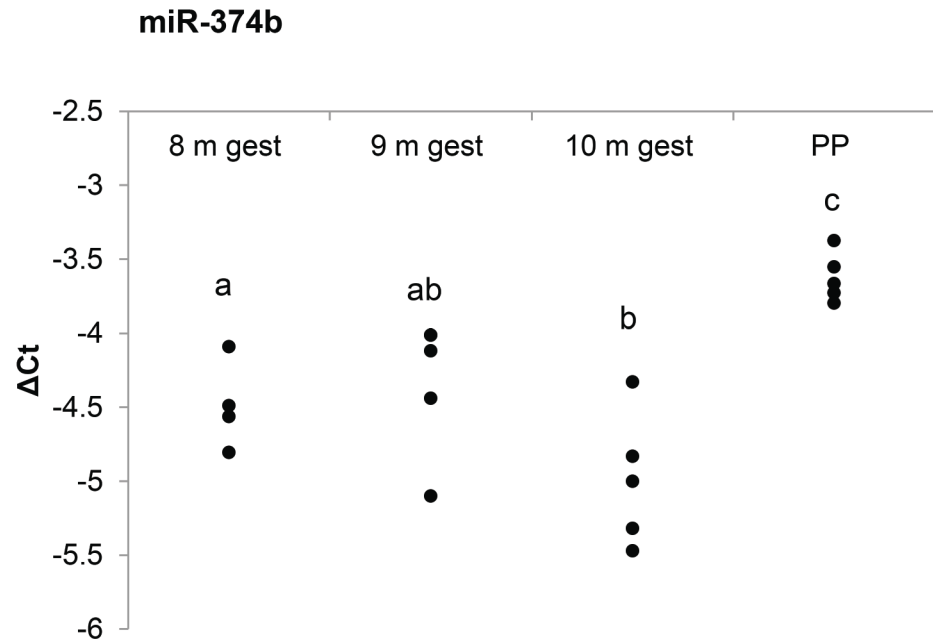


Fig 1. Differential expression of miR-374b through late gestation. Expression of circulating miRNAs was analyzed using quantitative PCR through late gestation (8, 9 or 10 months) and post-partum (PP). One miRNA, miR-374b, was found to be differentially regulated. Data shown represent the ΔCt value (dCt), calculated as $\Delta Ct = Ct_{(target)} - Ct_{(mean\ of\ sample)}$, with each dot representative of a separate sample. A lower ΔCt value indicates a higher expression level; for each 1-point reduction in ΔCt , an approximately 2-fold increase in expression can be assumed. Significantly different samples are indicated by varying superscripts.

<https://doi.org/10.1371/journal.pone.0175045.g001>

($P < 0.05$; Fig 1). Additionally, the expression level of miR-374b at 10 m gestation was up-regulated compared to 8 m gestation. When miR-374b was analyzed with mirDB, 247 putatively targeted mRNAs with a target prediction score of ≥ 80 were identified (S2 File). These mRNAs are hypothesized to be involved in 261 canonical pathways; the top twenty pathways being presented in Table 1, with a full list of canonical pathways found in S3 File. The top pathways included axonal guidance signaling, TGF- β signaling, insulin receptor signaling, regulation of the epithelial-mesenchymal transition pathway, relaxin signaling and estrogen receptor signaling.

We identified four miRNAs which had altered expression between pregnant and non-pregnant mares, including two miRNAs which were up-regulated during pregnancy (miR-204b, miR-486-5p) and two which were down-regulated (miR-454, miR-133b; Fig 2). Post-hoc analysis revealed that each stage of gestation (8 m, 9 m, 10 m and post-partum) was significantly different than diestrus for each of these miRNAs.

The number of predicted target mRNAs for each miRNA ranged from 59 (miR-486-5p) to 271 (miR-454; S2 File). Twenty-nine mRNAs were the predicted targets of more than a single miRNA. Intriguingly, 23 / 29 of these shared mRNA targets were targeted by both an up-regulated miRNA and a down-regulated miRNA, although this may be simply due to the relative number of predicted targets for each miRNA.

The mRNA targets of miRNAs differentially regulated in pregnancy were analyzed to determine their putative role in canonical pathways by Ingenuity Pathway Analysis, with a total of 332 pathways predicted (S3 File). The top twenty pathways are presented in Table 2. These pathways are primarily signaling pathways, with 17 of the top 20 pathways representing signaling pathways, including the STAT3 pathway, prolactin signaling, GnRH signaling, PI3/AKT

Table 1. Top twenty pathways predicted for the miRNA differentially regulated through late gestation (miR-374b).

| Ingenuity Canonical Pathway | -log (p-value) |
|---|-----------------------|
| VDR/RXR Activation | 4.28 |
| tRNA Splicing | 2.93 |
| Axonal Guidance Signaling | 2.39 |
| TGF- β Signaling | 2.31 |
| Insulin Receptor Signaling | 2.1 |
| GABA Receptor Signaling | 2.01 |
| Regulation of the Epithelial-Mesenchymal Transition Pathway | 2.01 |
| ErbB2-ErbB3 Signaling | 1.97 |
| Relaxin Signaling | 1.96 |
| Paxillin Signaling | 1.89 |
| Transcriptional Regulatory Network in Embryonic Stem Cells | 1.86 |
| Neurotrophin/TRK Signaling | 1.81 |
| PTEN Signaling | 1.76 |
| Protein Kinase A Signaling | 1.68 |
| G-Protein Coupled Receptor Signaling | 1.68 |
| Prolactin Signaling | 1.68 |
| Valine Degradation I | 1.67 |
| Estrogen Receptor Signaling | 1.63 |
| Neuregulin Signaling | 1.63 |
| GADD45 Signaling | 1.63 |

<https://doi.org/10.1371/journal.pone.0175045.t001>

signaling and JAK/stat signaling. Four of the pathways were present in the top 20 pathways for both miRNAs changing through late gestation, as well as for miRNAs changing between pregnant and non-pregnant samples. These duplicated pathways included the epidermal growth factor receptor family (ErbB2-ErbB3 signaling), the insulin receptor signaling pathway, the prolactin signaling pathway and the phosphatase and tensin homolog (PTEN) signaling pathway.

Discussion

We have identified, for the first time, pregnancy-associated circulating miRNAs in the late-pregnant mare. Due to their presence in peripheral circulation, these miRNAs are easily assessed with a simple blood test. Although their diagnostic ability is still being evaluated, they have potential to be used as biomarkers for the assessment of gestational well-being.

With any study on miRNAs, it is important to note the type of sample used, as well as the methodology employed for extraction and evaluation. This is particularly true for blood, as results vary significantly depending on whether plasma, serum, whole blood or exosomes are being targeted [17]. We chose to collect whole blood samples in PAXgene tubes because there is evidence that storing blood in RNA specific storage kits such as PAXgene tubes maintains the integrity of the RNA more effectively than serum stored at -80°C [18], as well as simplifying collection procedures. That said, there are downfalls of analyzing the miRNA composition of whole blood as compared to serum, plasma or exosomes. Whole blood contains not only miRNAs from the target tissues, but also miRNAs from each individual cellular components of blood including erythrocytes [19], leukocytes [20] and platelets [21]. The miRNAs derived from the cellular components of blood provide background noise, making it more difficult to identify changes in placental-derived miRNAs. Moreover, without separating leukocyte-

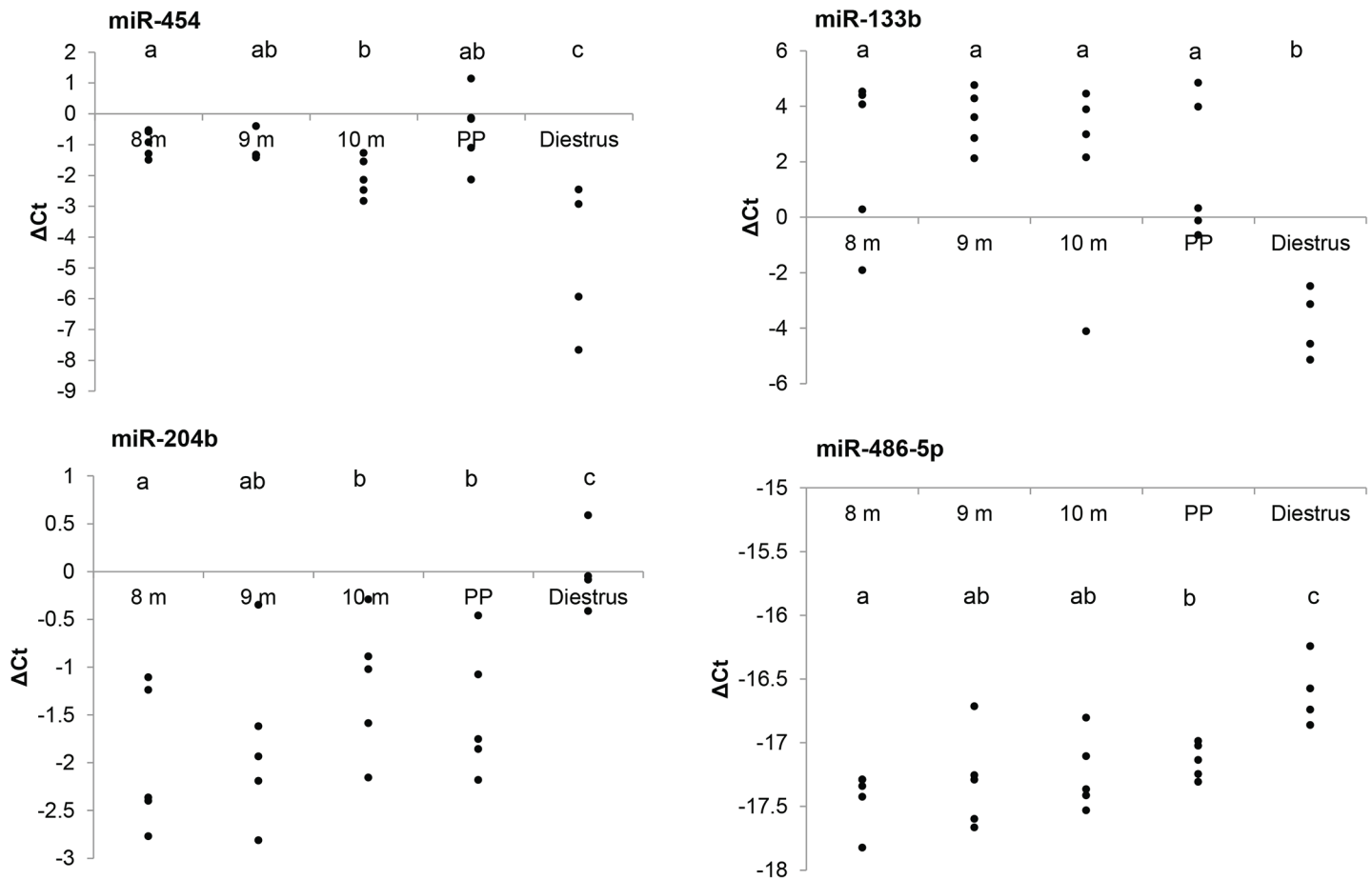


Fig 2. Differential expression of circulating miRNAs in pregnant mares. Expression of circulating miRNAs was analyzed using quantitative PCR to identify miRNAs with significantly different expression during pregnancy. Samples were grouped into pregnant (8, 9, 10 m gestation, postpartum (PP)) or non-pregnant (diestrus) samples and analyzed by one-way ANOVA corrected for false discovery rate (Benjamini-Hochberg; $P < 0.05$), with post-hoc analysis performed by student's t-test ($P < 0.1$). Data were normalized by $\Delta Ct = Ct_{(target)} - Ct_{(mean\ of\ the\ sample)}$, with each point representing the ΔCt for an individual mare. A lower ΔCt value indicates a higher expression level; for each 1-point reduction in ΔCt , an approximately 2-fold increase in expression can be assumed. Significantly different samples are indicated by varying superscripts.

<https://doi.org/10.1371/journal.pone.0175045.g002>

derived miRNAs from placental-derived miRNAs, we may be inadvertently biasing our samples towards inflammatory miRNAs rather than placenta-specific targets.

Of the four miRNAs differentially regulated during pregnancy, two were down-regulated. This is a significantly larger proportion than previously reported in human literature which has primarily focused on miRNAs with increasing expression during gestation [22–25]. This discrepancy may be due to study design, as one human study identified highly abundant miRNAs from the placenta, then looked for their presence in circulation [22], while another only reported up-regulated miRNAs [25] in a search for pregnancy-specific miRNAs. This difference in methodology is interesting in part due to the differences in expression we noted between our up- and down-regulated miRNAs. Within these four miRNAs, much larger changes in expression were seen in the down-regulated miRNAs. Looking at miR-133b, large fold-changes between pregnancy and diestrus were consistently noted, ranging from a 45 X decrease during the post-partum period to a 164 X decrease at 9 m gestation. The other down-regulated miRNA (miR-454) was 12.9 X lower on average (range of 6.5–19.2 X). The two up-regulated miRNAs (miR-204b and miR-486-5p) had an average increase of only 2.4 X (range–

Table 2. Top twenty pathways predicted for miRNAs differentially regulated during pregnancy (miR-133b, miR-204b, miR-454, miR-486-5p).

| Ingenuity Canonical Pathways—Pregnant Mares | -log (p-value) |
|---|----------------|
| STAT3 Pathway | 5.35 |
| PPARα/RXRα Activation | 4.99 |
| Prolactin Signaling | 4.76 |
| PTEN Signaling | 4.41 |
| IGF-1 Signaling | 4.32 |
| Glioblastoma Multiforme Signaling | 4.14 |
| JAK/Stat Signaling | 4.05 |
| CTLA4 Signaling in Cytotoxic T Lymphocytes | 4.03 |
| PAK Signaling | 3.94 |
| Glioma Signaling | 3.59 |
| GNRH Signaling | 3.52 |
| ErbB2-ErbB3 Signaling | 3.41 |
| EGF Signaling | 3.41 |
| Renin-Angiotensin Signaling | 3.24 |
| Insulin Receptor Signaling | 3.2 |
| Mouse Embryonic Stem Cell Pluripotency | 3.15 |
| PI3K/AKT Signaling | 3.14 |
| GDNF Family Ligand-Receptor Interactions | 3.05 |
| Telomerase Signaling | 3.05 |
| Non-Small Cell Lung Cancer Signaling | 3.01 |

<https://doi.org/10.1371/journal.pone.0175045.t002>

1.44–3.96 X; Fig 2). This is not surprising biologically, as miRNAs are responsible for the down-regulation of mRNA, and mRNA expression increases overall during pregnancy. Although it is difficult to make broad conclusions based on only four differentially regulated miRNAs, it is an intriguing trend.

The two miRNAs we identified as down-regulated during late gestation have not previously been identified as being important during pregnancy, although this could be largely due to prior study design in humans. Out of our up-regulated miRNAs, miR-486-5p has not been previously reported to be pregnancy-related, despite being the most highly expressed miRNA within our samples. Overall, expression levels of miR-486-5p increased by an average of 1.6-fold during pregnancy. Our other up-regulated miRNA, miR-204b, has an identical mature sequence to hsa-miR-204, which has been shown to be up-regulated in pre-eclampsia [26], potentially due to its role in suppressing trophoblast-like cell invasion [27].

Although our down-regulated miRNAs, miR-133b and miR-454, are certainly not pregnancy specific, their down-regulation during pregnancy allows for an increase in the translation of numerous mRNAs, including X-linked RNA binding motif protein (RBMX) which is implicated in regulation of gene transcription and alternative splicing, as well as repairing double-stranded DNA breaks [28, 29] and DEAD (Asp-Glu-Ala-Asp) box helicase 6 (DDX6) which maintains adult progenitor cell function [30].

Together, the mRNAs targeted by the pregnancy-related miRNAs are predicted to be a part of 332 canonical pathways by Ingenuity Pathway Analysis (S3 File). Included in the top twenty predicted pathways are the STAT3 pathway, JAK/stat signaling, prolactin signaling, PI3K/AKT signaling, GnRH signaling, and mouse embryonic stem cell pluripotency markers. The purported effect of these miRNAs on the prolactin and GnRH signaling pathways is interesting due to the essential role of these pathways in pregnancy.

Although GnRH hasn't been studied extensively in late pregnancy, its ability to stimulate gonadotropins and with that, steroid hormones such as estrogens and progestogens, is well established [31]. Prolactin is perhaps best known for its role in lactation [32]; however, it is also responsible for activating several other pathways such as the JAK-STAT pathway, including STAT3 [33, 34]. The function of STAT3 is well established in pregnancy, including involvement with embryonic implantation [35, 36], angiogenesis [37], and parturition [38, 39].

PI3K/AKT signaling has been shown to play a role in trophoblast migration [40] and placental resource allocation [41], as well as being implicated in embryonic neurogenesis and angiogenesis [40, 42]. Many of these pathways also work in conjunction with one another; the PI3K and prolactin signaling pathways have been shown to increase pancreatic islet mass and sensitivity to glucose during pregnancy [43]; likely influencing insulin receptor signaling, another affected pathway, simultaneously.

Although we have some information about the likely function of these differentially expressed miRNAs, we do need to proceed with caution. Many of our miRNAs did not exist in the Ingenuity Pathway Analysis database, so to get an equivocal analysis we derived predicted mRNA targets using miRDB, a human dataset [15]. Since mRNAs are not identical across species, it is possible that some of these mRNAs are not present in the horse or that they do not contain the miRNA target sequences. However, miRNA annotation is highly conserved and identical miRNAs are assigned the same number regardless of organism, increasing the likelihood of cross-species applicability [44]. Additionally, we only selected mRNA targets with a predicted target score of 80 or above, a level which the designers considered "very probable" to be a valid target [15]. Additional caution should be exercised with the designation of pathways by Ingenuity Pathway Analysis. Many of the designated pathways contain overlapping molecules, so it is possible for multiple pathways to be considered influenced even though it is unlikely for many of those same pathways to be present or applicable to the work. For example, it is unlikely that our miRNAs are affecting glioblastoma multiform signaling, despite this being the top predicted pathway for pregnancy-related miRNAs. Despite these concerns, most of the predicted pathways are not unexpected and provide valuable information about the biology of these differentially regulated miRNAs.

In conclusion, we have identified eight circulating miRNAs which changed significantly during gestation. The pregnancy-specific miRNAs are predicted to target mRNAs involved in signaling pathways, particularly those which are involved in placentation, angiogenesis and endocrinology of pregnancy. This study not only identifies potential biomarkers for placental function in late gestation, it also provides information about their putative targets and what pathways may be affected during these periods.

Supporting information

S1 File. Raw and normalized Ct values for qPCR.

(XLSX)

S2 File. Putative mRNA targets. Putative mRNA targets for individual miRNAs as predicted by miRDB [15]. Only mRNAs with a predicted target score of ≥ 80 are included. Includes tab for shared mRNAs which were targeted by multiple miRNAs.

(XLSX)

S3 File. Predicted canonical pathways. Ingenuity Pathway Analysis (IPA) was used to predict canonical pathways affected by putative mRNA targets of differentially regulated miRNAs. Putative mRNA targets were identified using miRDB [15], and mRNAs with a predicted target

score of ≥ 80 were pooled and analyzed via IPA core analysis.
(XLS)

S1 Table. Primer sequences. Sequences of 178 primers used for qPCR analysis.
(DOCX)

Acknowledgments

The authors would like to thank Tracy Mask for her technical assistance with the PCR analysis.

Author Contributions

Conceptualization: SCL BAB.

Formal analysis: SCL BAB.

Funding acquisition: MHTT ELS BAB.

Investigation: SCL.

Methodology: SCL BAB KES JEB.

Project administration: SCL BAB JEB.

Resources: BAB JEB IFC.

Supervision: BAB.

Validation: SCL BAB.

Visualization: SCL.

Writing – original draft: SCL.

Writing – review & editing: SCL KES JEB IFC MHTT ELS BAB.

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