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Differential expression of microRNAs with progression of gestation and inflammation in the human chorioamniotic membranes

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Abstract

Objective—The aim of this study was to identify differential expression of microRNAs (miRNAs) in chorioamniotic membranes with advancing gestation, labor, and inflammation.

Study design—Expression profiles of 157 miRNAs in the chorioamniotic membranes were obtained from patients in the following groups: 1) term not in labor (n=10); 2) term in labor (n=10); 3) preterm labor with histologic chorioamnionitis (n=9); and 4) without histologic chorioamnionitis (n=10).

Results—More than 95% of the miRNAs screened were expressed. Gestational age-dependent changes in expression were observed for 13 miRNAs. No differences in miRNA expression were observed between women without labor and women in labor. Membranes with chorioamnionitis displayed increased expression of miR-223 and miR-338. Gene Ontology analysis of genes targeted by differentially expressed miRNAs revealed enrichment for specific biological process categories.

Conclusion—Chorioamniotic membranes with advancing gestational age and chorioamnionitis are associated with the differential expression of a subset of miRNAs.

Keywords

chorioamniotic membranes; gestation; inflammation; labor; microRNA; real-time qRT-PCR

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Introduction

The human chorioamniotic membranes are involved in a variety of physiological and pathological processes during pregnancy, such as accommodation and protection of the developing fetus, parturition, and response to intra-amniotic infection. Infection and inflammation of the amniotic cavity are often accompanied by histologic chorioamnionitis,[1] which is associated with both preterm delivery and adverse perinatal outcome.[2] Although the structure of the chorioamniotic membranes does not substantially change after the first trimester,[3] gestational age-dependent changes in gene expression have been reported.[4] Functional genomic studies of the chorioamniotic membranes have shown that spontaneous labor at term[5;6] and intra-amniotic infection[6] are both characterized by unique gene expression signatures. Little is known, however, about the mechanisms involved in the regulation of gene expression in the chorioamniotic membranes.

MicroRNAs (miRNAs) are small, non-coding RNA molecules that are critical for the post-transcriptional regulation of gene expression.[7] Originally discovered in the nematode, *Caenorhabditis elegans*,[8] miRNAs inhibit translation or initiate degradation of target messenger RNA (mRNA) transcripts via complementary base-pairing.[9] Conservation of miRNAs within and across species, as well as co-evolution with mRNAs, suggest that miRNAs have had a significant impact on the evolution of protein-coding genes.10 Over 400 miRNAs have been identified in humans,11 and bioinformatics studies predict over one-third of human genes are regulated by miRNAs.12

miRNAs are being studied extensively, particularly in embryonic development. Mice and zebrafish lacking the miRNA processing enzyme Dicer are not viable past the early embryonic period.[13;14] Moreover, lack of Dicer in mice disrupts hair germ invagination, suggesting that miRNAs are critical for hair follicle morphogenesis.[15] A role for miRNAs in mammalian immunity has also recently been proposed.[16;17] miR-155 expression by macrophages is induced by the ligands of multiple Toll-like receptors.[16] In neutrophils, miR-146a and miR-146b target adapter molecules TRAF6 and IRAK1, which are essential for the downstream signaling of both cytokine receptors and Toll-like receptors.[17]

Because of the evidence supporting miRNA involvement in normal developmental processes as well as immune responses, we sought to determine whether miRNA expression in the human chorioamniotic membranes varied with advancing gestational age, spontaneous labor at term, and histologic chorioamnionitis.

Material and Methods

Study design

A cross-sectional study was conducted to examine the patterns of miRNA expression in chorioamniotic membranes obtained from patients in the following groups: 1) normal pregnancy at term not in labor and delivered by elective cesarean section ([TNL], n=10); 2) normal pregnancy with spontaneous labor at term ([TL], n=10); 3) spontaneous preterm labor and delivery without histologic chorioamnionitis ([PTL], n=10); and 4) spontaneous preterm labor and delivery with histologic chorioamnionitis ([PTL-HC], n=9). Normal pregnancy was defined by the absence of medical, surgical, or obstetrical complications. Preterm labor was defined by the presence of regular uterine contractions (at a frequency of at least 2 every 10 minutes) associated with cervical changes and leading to preterm delivery before 37 completed weeks of gestation. Histologic chorioamnionitis was diagnosed by the presence of neutrophilic infiltration on the chorioamniotic membranes. All neonates were appropriate for gestational age (birth weights 10^{th}-90^{th} percentile),[18] and none of the individuals who delivered at term had evidence of histologic chorioamnionitis. Patients with premature rupture of membranes,

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multiple gestation, stillbirth, or fetal anomalies were excluded. All women provided written informed consent for the collection of clinical data and tissue samples under protocols approved by the Institutional Review Boards of both Wayne State University (Detroit, MI) and the National Institute of Child Health and Human Development of the National Institutes of Health (NIH/DHHS).

RNA isolation

Details of the materials and methods used in this study were previously reported[19] and will be briefly described. Snap-frozen chorioamniotic membranes stored at −80°C were used. Small RNAs (<200 nucleotides) were obtained using the mirVana RNA Isolation kit (Ambion, Austin, TX). Small RNA integrity was determined by polyacrylamide gel electrophoresis. Small RNA concentration was determined with the RNA 6000 Nano Assay on the 2100 Bioanalyzer (Agilent Technologies, Inc, Palo Alto, CA).

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) for miRNA assay

miRNA expression profiling of 157 human miRNAs was determined with the TaqMan MicroRNA Assays Human Panel–Early Access Kit (Applied Biosystems, Foster City, CA). A custom-designed TaqMan assay for 5S ribosomal RNA was used to normalize miRNA expression.

Statistical analysis

miRNA expression profiles were measured to evaluate 3 processes: 1) gestational age (PTL [n=10]); 2) labor (TNL [n=10] vs TIL [n=10]); and 3) histologic chorioamnionitis (PTL [n=9] vs PTL-HC [n=9]). For the analysis of histologic chorioamnionitis, 9 cases from the PTL group were selected with gestational ages matched to cases in the PTL-HC group.

Generalized estimating equations (GEE) were used to model the real-time qRT-PCR data.20 For each miRNA, a GEE model was fit to estimate the effect of the covariate (obstetric condition or gestational age) on the observed expression using the gee package in R statistical software,21 specifying an exchangeable correlation structure (every pair of technical replicates has the same correlation). A *P* value was derived to assess the significance of the covariate into the model, and the resulting *P* values were adjusted using the false discovery rate (FDR) correction.[22] A corrected *P* value of <0.05 was considered statistically significant.

miRNA target prediction and Gene Ontology analysis

Predicted targets of miRNAs differentially expressed in this study were determined using miRBase Targets.[23] Gene Ontology (GO) was employed to categorize genes by cellular component, molecular function, and biological process.[24] We used Onto-Express25–27 to tally the number of mRNA targets in each GO category and compare it with the expected number of targets found in the GO category. The union of target genes predicted by miRBase for all assayed miRNAs was used as a reference. Significant differences from the expected number of genes were calculated with the assumption of a hypergeometric distribution; *P* values were adjusted with the false discovery rate correction[22] based on the number of GO categories that were tested. A corrected *P* value of <0.05 was considered statistically significant.

Results

Demographic characteristics of the study population are summarized in Table 1. Term chorioamniotic membranes expressed 153 out of 157 miRNAs, and preterm chorioamniotic membranes expressed 152 out of 157 miRNAs (expression detected in at least 50% of samples).

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The effect of gestational age on miRNA expression was evaluated in the chorioamniotic membranes of 10 patients with no evidence of histologic chorioamnionitis who delivered between 26.3–35.9 weeks of gestation. The expression of 13 miRNAs decreased with advancing gestational age (Figure 1A; Table 2), and varied between a 4.8-fold (miR-199b) and a 1.6-fold (miR-330) change per month. No significant differences were observed between the miRNA expression profiles of women at term not in labor and those in spontaneous labor after adjustment for multiple comparisons. Evaluation of preterm membranes with and without chorioamnionitis identified differential expression of 2 miRNAs (Figure 1B). The expression of miR-223 and miR-338 was increased (37-fold and 24-fold, respectively) in the presence of chorioamnionitis.

GO analysis performed on gene target lists of miRNAs differentially expressed with chorioamnionitis revealed enrichment for biological process categories. Processes with significantly higher than expected representation in the gene target lists included transcription from RNA polymerase II promoter for miR-223 (Table 3) and electron transport for miR-338 (Table 4). Genes involved in parturition were enriched in the target list of miR-338 (group IVB phospholipase A2 and corticotropin-releasing hormone receptor 1).

Comment

The principal findings of this study are: 1) miRNAs are abundantly expressed in the chorioamniotic membranes; 2) distinct miRNA expression patterns are associated with advancing gestation; and 3) specific miRNAs are differentially expressed with histologic chorioamnionitis.

The temporal regulation of miRNA expression during animal development has been closely investigated. For example, the expression of let-7a, a member of the let-7 miRNA family, is critical for developmental timing in the model organisms nematode[28] and mouse.[29] In this study, let-7 miRNAs did not demonstrate differential expression with advancing gestation. However, 13 miRNAs displayed decreased expression with advancing gestation, which suggests a functional involvement of miRNAs in chorioamniotic membranes by reducing the translational inhibition of multiple mRNA targets.

GO analysis of genes targeted by miRNAs differentially expressed with chorioamnionitis (miR-223 and miR-338) revealed enrichment for specific biological process categories. Interestingly, miR-338 was also found to decrease with advancing gestation, and is predicted to target genes involved in the GO biological process of parturition (group IVB phospholipase A2 and corticotropin-releasing hormone receptor 1 [CRHR1]). Because CRH increases exponentially throughout gestation, mainly through placental production, it has been proposed as a critical factor in determining the timing of parturition.[30] CRHR1, the primary receptor for corticotropin-releasing hormone (CRH), is expressed by amnion, chorion, and decidua,31 as well as reproductive tissues such as the cervix.32 Locally, CRH is a potent vasodilator that can induce prostaglandin33 and matrix metalloproteinase34 production by the chorioamniotic membranes. Since miR-338 may interact with CRHR1 in the membranes, additional studies are needed to evaluate the impact of miR-338 on CRHR1 function.

The target list of miR-223 was enriched for genes involved in fundamental biological processes, as well as several immunity-related processes. Chorioamniotic membranes play a complex dual role of protecting the fetus from both foreign pathogens and the maternal “host” immune response. Several studies have shown that chorioamniotic membranes are involved in the recognition and response to infection. For example, membranes with chorioamnionitis display increased expression of Toll-like receptors-2 and 4, which respond to Gram-positive and Gram-negative bacteria, respectively.[35] Selected genes in the target list of miR-223 (SPINK5,

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TGFB2, and IFNB1) are involved in the GO biological processes of negative regulation of immune response and anti-inflammatory response. The over-representation of immune-related biological process categories in the target list of miR-223 suggests a function for this miRNA in the regulation of chorioamnionitis-related inflammation.

A strength of this study is that the differential expression of 157 human miRNAs was measured using real-time qRT-PCR, which is considered the most sensitive and specific RNA quantification method. In addition, a correction for the multiple testing of 157 miRNAs was performed by employing the false discovery rate, which contributed to a robust and reliable assessment of differential miRNA expression. A constraint of this study, on the other hand, is that the real-time qRT-PCR method did not allow for a more comprehensive screening of all human miRNAs currently identified. This limitation may partly explain why no differences in expression were observed between women at term in labor and without labor.

This study reports, for the first time, the expression profiles of miRNA in human chorioamniotic membranes with advancing gestation and chorioamnionitis. The miRNAs differentially expressed in these conditions provide relevant targets for further investigation. miRNA inhibition and overexpression using *in vitro* and *in vivo* experimental models are required to confirm functionally relevant mRNA targets and to assess the biological significance of differential miRNA expression in chorioamniotic membranes with advancing gestation and chorioamnionitis.

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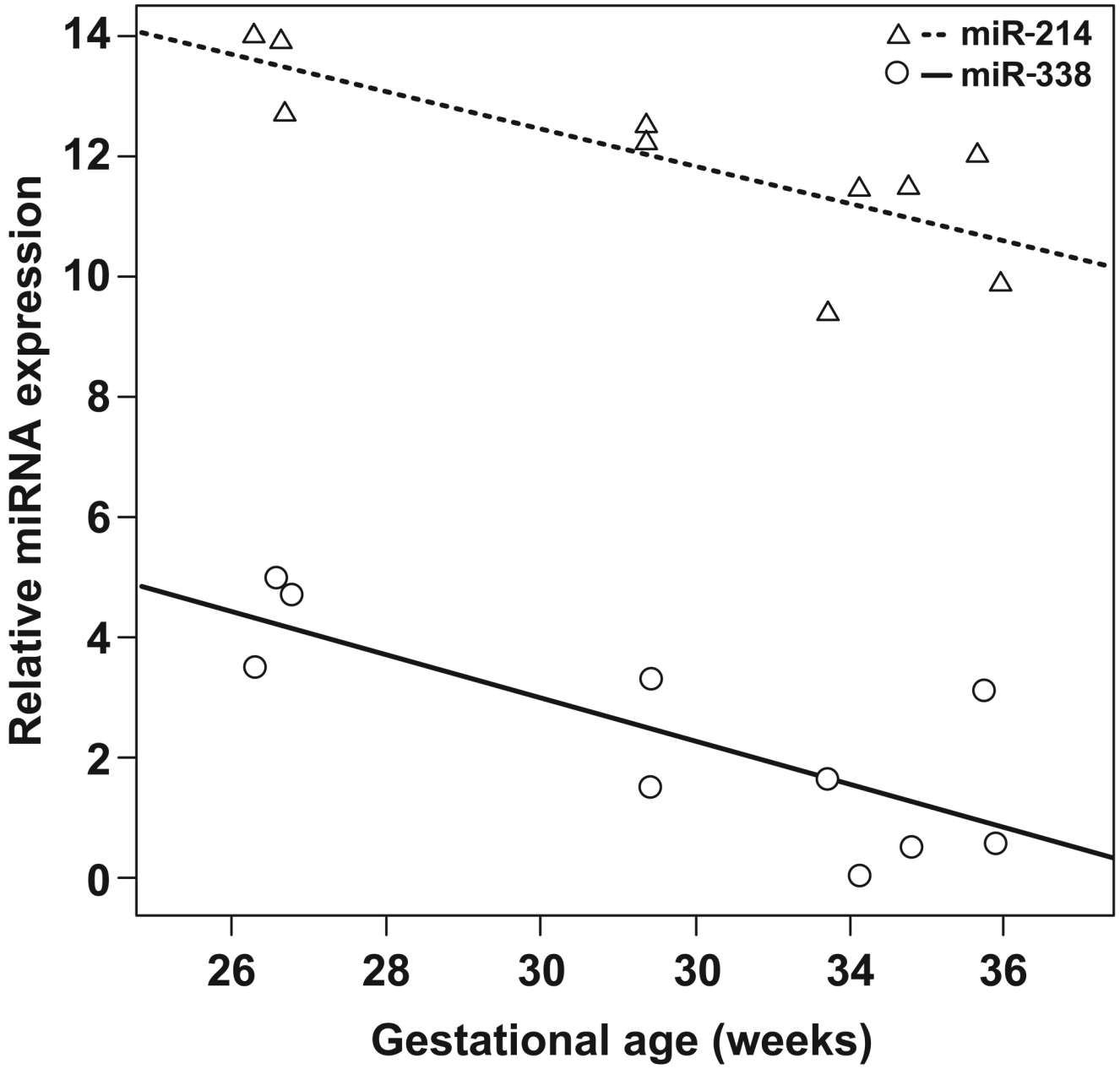

Figure 1. Differential expression of microRNAs with advancing gestational age and histologic chorioamnionitis

A, Changes in miR-214 and miR-338 expression as a function of gestational age analyzed within the group of 10 cases with preterm labor without histologic chorioamnionitis. 13 microRNAs displayed decreasing expression with advancing gestation. This figure illustrates 2 examples. Multiple adjustment for the testing of 157 miRNAs was performed (all $P < .05$). The y-axis represents units of delta Ct (Ct5S – CtmiRNA), with an arbitrary zero point, so that each unit measures a 2-fold change. The x-axis represents gestational age in weeks. **B**, Differential expression of microRNAs with histologic chorioamnionitis. Box-and-whisker plots of microRNAs differentially expressed between preterm labor cases with and without histologic chorioamnionitis after multiple units of delta Ct (Ct5S – CtmiRNA), with an arbitrary

zero point, so that each unit measures a 2-fold change. The fold change is displayed below each microRNA name.

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Table 1

Patient demographics and clinical characteristics

Characteristics	Term not in labor (n=10)	Term in labor (n=10)	Preterm labor without chorioamnionitis (n=10)	Preterm labor with chorioamnionitis (n=9)
Maternal age (y)	24 (19–34)	24 (20–34)	25 (18–36)	24 (19–32)
Parity	2 (0–4)	2 (0–5)	1 (0–7)	1 (0–5)
Black ethnicity	9 (90)	9 (90)	8 (80)	8 (89)
Gestational age at delivery (wks)	39.4 (37.0–41.7)	39.3 (37.0–41.9)	32.6 (26.3–35.9)	33.7 (25.3–35.9)
Birthweight (g)	3225 (2940–3915)	3150 (2800–3595)	1848 (870–2810)	1785 (690–2605)

Values expressed as median (range) or n (%).

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Table 2

MicroRNAs demonstrating changes in expression with advancing gestational age

microRNA	Fold change per month	r^2
miR-199b	4.8	0.56
miR-373	3.5	0.60
miR-218	3.4	0.50
miR-154	3	0.51
miR-338	2.7	0.62
miR-198	2.6	0.44
miR-214	2.4	0.64
miR-370	2.4	0.55
miR-213	2.4	0.63
miR-107	2.3	0.51
miR-199a	2.1	0.41
miR-222	1.9	0.49
miR-330	1.6	0.40

All $P < 0.05$

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Table 3

Gene Ontology analysis of miR-223 targets

Biological process category	Genes targeted by miR-223	Genes targeted by all miRNAs*	Predicted targets
Transcription from RNA polymerase II promoter	15	155	BTF3, MEF2C, HLX1, TCFL5, CBFB, TCERG1, NFE2L3, LMO4, FOXO3A, PARP1, ATBF1, ECD, GTF2H4, RFXANK, CREB3
Protein modification	9	70	RPN2, BAG1, C16orf33, PIGH, TTLL9, PAM, SUMO1, PCMT1, DDI1
Protein targeting	7	29	SRP54, SEC61B, SRP19, GABARAP, SEC61G, SIL1, RRBP1
Transcription from RNA polymerase III promoter	4	14	SSB, TROVE2, GTF3C2, GTF3C1
Negative regulation of immune response	2	3	SPINK5, TGFB2
Superoxide release	2	3	DUOX1, DUOX2
Anti-inflammatory response	2	5	SPINK5, IFNB1
Hydrogen peroxide biosynthesis	2	2	DUOX1, DUOX2
Hydrogen peroxide catabolism	2	7	DUOX1, DUOX2

All $P < 0.05$

* miRNAs studied in this analysis.

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Table 4

Gene Ontology analysis of miR-338 targets

Biological process category	Genes targeted by miR-338	Genes targeted by all miRNAs*	Predicted targets
Electron transport	22	253	CYP27B1, DUOX1, HIF1A, BLVRA, NNT, FTSJ3, PYCR2, NFIA, CYP3A5, MICAL3, NDUFV1, FDXR, DHX34, SURF1, TXNRD2, COX4I1, PDIA3, GCDH, P4HB, PAOX, CYP3A7, GPD2
Protein catabolism	4	17	FBXO17, PSMC6, AFG3L2, PRSS16
Fibrinolysis	3	7	TMPRSS6, F12, PLG
Protein secretion	3	11	ARFGAP3, GNAS, SEC61A2
Carbohydrate transport	3	12	SLC2A14, SLC2A10, SLC2A3
L-cystine transport	2	2	SLC3A1, CTNS
Tissue regeneration	2	4	NINJ2, APOA5
Homeostasis	2	5	PKHD1, HIF1A
Parturition	2	7	CRHR1, PLA2G4B

All $P < 0.05$

* miRNAs studied in this analysis.