MicroRNA-548 regulates high mobility group box 1 expression in patients with preterm birth and chorioamnionitis

Ga Hyun Son

Department of Medicine

The Graduate School, Yonsei University

MicroRNA-548 regulates high mobility group box 1 expression in patients with preterm birth and chorioamnionitis

Ga Hyun Son

Department of Medicine

The Graduate School, Yonsei University

MicroRNA-548 regulates high mobility group box 1 expression in patients with preterm birth and chorioamnionitis

Directed by Professor Young Han Kim

The Doctoral Dissertation submitted to the Department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Ga Hyun Son

June 2019

This certifies that the Doctoral Dissertation of Ga Hyun Son is approved.

Thesis Supervisor: Young Han Kim

Thesis Committee Chair: SiHyun Cho

Thesis Committee Member: Chae Gyu Park

Thesis Committee Member: Han Sung Kwon

Thesis Committee Member: Joo Young Kim

The Graduate School
Yonsei University

June 2019

ACKNOWLEDGEMENTS

I would like to express deepest gratitude to my supervisor Prof. Kim, Young Han for his full support, expert guidance, understanding and encouragement throughout my study and research. Without his incredible patience and timely wisdom and counsel, my thesis work would have been a frustrating and overwhelming pursuit. I also express my warmest gratitude to my other supervisors, Prof. Chae Gyu Park. His guidance and supervision have been essential during this work. In addition, I express my appreciation to Prof. Han Sung Kwon for having served on my thesis committee. Their thoughtful questions and comments were valued greatly.

I would also like to thank Prof. Joo Young Kim for dedicated guidance and support of my coursework and academic research. Special thanks go to Prof. SiHyun Cho for his hearty encouragement, support, and sharp supervision of my study.

Finally, I would like to thank my family for their unconditional love and support during the last years. I would not have been able to complete this thesis without their continuous love and encouragement.

Thank you.

Ga Hyun Son

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

MicroRNA-548 regulates high mobility group box 1 expression in patients with preterm birth and chorioamnionitis

Ga Hyun Son

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Young Han Kim)

Infections and inflammations of the amniotic cavity are often accompanied by histologic chorioamnionitis, which is associated with both preterm delivery and adverse perinatal outcome. High mobility group box 1 (HMGB1) is a prototypic alarmin. It plays an important role in the pathogenesis of inflammatory process in pregnant women, and is associated with spontaneous preterm birth. This study was conducted to compare the levels of HMGB1 in amniotic fluid and amnion membranes in women with chorioamnionitis/intra-amniotic inflammation to those in healthy controls. We also aimed to determine the expression of MicroRNA-548 (miR-548) cluster in amnion membrane, and to elucidate its involvement in regulating HMGB1 expression and its function in human amniotic epithelial cells (hAECs). Levels of HMGB1 in amniotic fluid (AF) were compared between women with intra-amniotic inflammation (n=34) and normal controls (n=14). We also determined HMGB1 expression levels in amnion membranes of women with chorioamnionitis (n=6) against those in healthy controls (n=4). MiR-548 cluster was predicted to bind HMGB1 by a bioinformatics analysis. To investigate the causal relationship between miR-548 cluster and HMGB1, a repressed and forced expression assay in hAECs was performed. The levels AF HMGB1 were significantly higher in patients with intra-amniotic inflammation than in those without inflammation. HMGB1 expression was also increased in amnion membranes from women with preterm birth and chorioamnionitis as compared to normal controls. MiR-548 cluster was significantly under-expressed in amnion membranes from patients with chorioamnionitis than in normal term controls. Repressed expression of miR-548 up-regulated HMGB1 expression in hAECs and increased its release from hAECs. Moreover, forced expression of miR-548 suppressed HMGB1 and

inflammatory cytokines in hAECs, which increased when treated with lipopolysaccharide. In conclusion, intra-amniotic inflammation suppresses miR-548, which increases HMGB1 expression in the amnion membrane and HMGB1 release. Moreover, miR-548 can alter the inflammatory responses in hAECs. MiR-548 might be involved in the pathogenesis of preterm birth and chorioamnionitis by regulating HMGB1.

Key words : chorioamnionitis, high mobility group box 1, intra-amniotic inflammation, microRNA-548, preterm birth

MicroRNA-548 regulates high mobility group box 1 expression in patients with preterm birth and chorioamnionitis

Ga Hyun Son

Department of Medicine The Graduate School, Yonsei University

(Directed by Professor Young Han Kim)

I. INTRODUCTION

Preterm birth occurs between fetal viability and 37 completed weeks of gestation.[1] The definition of viability is controversial because of the increasing frequency of survival at progressively lower gestational ages. Most countries define it as a lower limit of 20 to 22 weeks, but this varies.[2] Worldwide, approximately 1.1 million neonates die from prematurity-related complications.[3] Term and preterm labor share common pathways, which include increased uterine contractility, cervical ripening, and membrane rupture leading to fetal prematurity and damage.[4] However, whereas term birth results from physiologic activation of these common pathways, preterm birth results from a pathologic activation of the components of the common pathways via similar or alternative mechanisms.[5,6] The common pathways of parturition includes anatomic, biochemical, immunologic, endocrinologic, and clinical changes.[5] Although the anatomic and clinical events have been studied in detail, the biochemical, immunological, and endocrine events are still incompletely

understood.[7] Prostaglandins are viewed as crucial mediators for the onset of labor, and intra-amniotic infection and inflammation are central to preterm birth.[8-13]

During pregnancy, fetal chorioamniotic membranes are involved in a variety of physiological and pathological processes, such as accommodation and protection of the developing fetus, parturition, and response to intra-amniotic infections.[14] Infections and inflammations of the amniotic cavity are often accompanied by histologic chorioamnionitis, which is associated with both preterm delivery and adverse perinatal outcome.[15] Intra-amniotic inflammation can be due to microbial invasion of the amniotic cavity or other mechanisms in which necrosis or cellular stress induces the release of mediators that activate the innate immune system.[16-18] Damage associated molecular patterns (DAMPs), including S100 calcium binding protein B (S100B),[19] heat shock proteins,[20] interleukin (IL)-1α,[20-22] and high mobility group box 1 protein (HMGB1),[23,24] are endogenous proinflammatory and pro-oxidative stress molecules that can induce the inflammatory process in patients without demonstrable infection (sterile inflammation).[20,23,24] Acting through Toll-like receptor 2 and 4 (TLR2 and TLR4), and by the receptor for advanced glycation end products (RAGE), DAMPs recruit inflammatory cells, which in turn amplify the innate immune response, thus favoring cytokine activation.[25] It was reported that the RAGE-DAMP system is present in women with preterm birth and

intra-amniotic inflammation. Activation of the RAGE-DAMP system correlates with the degree of inflammation and oxidative stress damage in amnion epithelial, decidual, and extravillous trophoblast cells.[26]

HMGB1 is a prototypic alarmin that has a central role in the pathogenesis of both sterile and infectious inflammation.[27-34] Elevated concentrations of HMGB1 may reflect engagement of DAMP-induced inflammation.[27,30,33,35,36] Although primarily located in the cell nucleus, HMGB1 can be secreted by innate immune cells in response to pathogenic products and can be released by injured or dying cells.[37] The biological activity of the non-histone nuclear protein HMGB1 depends on its location, context and post-translational modification. Inside the cell, HMGB1 binds DNA and modulates chromosomal architecture, however, with cell activation, injury or death, HMGB1 can translocate outside of the cell. There, HMGB1 can serve as a DAMP or alarmin to stimulate the innate immune system either by itself or as part of complexes with cytokines, as well as other exogenous and endogenous molecules. HMGB1 is a DNA-binding protein that is abundant in the cell nucleus, although it can also be cytoplasmic. As a nuclear molecule, HMGB1 regulates transcription, repair and recombination through exerting effects on chromosomal architecture. Although predominantly located in the nucleus, HMGB1 can also translocate to the cytoplasm, as well as the extracellular space. This process occurs in several distinct settings: activation of macrophages and

other immune cells by cytokines and TLR ligands; necrosis; apoptosis; and hypoxia and ischemia/reperfusion injury of parenchymal cells. Depending on the inducing stimulus, the physical and/or chemical form of HMGB1 can vary. HMGB1 can serve as an early mediator in the context of sterile inflammation, with release occurring either as a consequence of acute cellular stress, such as hypoxia, or as a necrosis. Moreover, numerous immune and nonimmune cell types can release HMGB1 by both passive and active mechanisms. Within the cell, the protein exerts diverse and compartment-specific functions, whilst outside the cells, its DAMP-like functions range from proinflammatory to tissue regeneration, and even feedback suppression of cellular responses. This wide range of extracellular activities arises from different HMGB1 isoforms being able to mediate signaling through at least 10 different receptors.

Buhimschi et al. reported that HMGB1 levels are increased in the amniotic fluid of women with intra-amniotic inflammation and preterm birth. Through explant experiments, the authors also showed that intra-amniotic HMGB1 might be released from the damaged amniochorion.[38] Additionally, intra-amniotic administration of HMGB1 was reported to induce spontaneous preterm labor and birth.[39] These findings suggested that intra-amniotic HMGB1 secreted from fetal membranes can induce premature labor and, therefore, may be involved in signaling parturition in the context of sterile intra-amniotic inflammation. The amnion is the innermost layer of the intra-amniotic cavity. Thus, we can

postulate that the amnion, rather than the chorion, plays a major role in response to changes in the amniotic cavity, such as intra-amniotic inflammation.[40] However, little is known about the role of amnion in preterm birth determined by intra-amniotic inflammation.

MicroRNAs (miRNAs) are a class of non-coding small RNAs ranging from 18 to 25 nucleotides in size, which are involved in post-transcriptional regulation of gene expression by mRNA degradation or translational repression.[41-43] MiRNA genes are most commonly transcribed by RNApolymerase II, and in some cases by RNA polymerase III.[41,42] MiRNAs can be found in the introns of protein-coding genes or as independent genes. Furthermore, a primary transcript can contain a single miRNA or multiple miRNAs that are processed out of the same transcript. Following initial processing by Drosha and DGCR8 in the nucleus, the premiRNA is exported to the cytoplasm where the miRNA hairpin is cleaved by Dicer resulting in a miRNA duplex. One of the RNA strands is loaded into the RNA-induced silencing complex (RISC) and guides this complex to the 3′-untranslated regions (3′UTRs) of target messenger RNAs (mRNAs) leading to repressed target protein expression.[43] This can occur from a variety of reported mechanisms including decreased mRNA stability due to deadenylation and uncapping, or via inhibition of translation. Approximately 500−1,000 miRNAs are expressed in human cells, and their expression signatures vary depending on

the tissue and cell types examined. At the molecular level, miRNAs have been shown to impact target gene expression anywhere from 1.2- to 4-fold, indicating that they do not function as on-off switches for genes, but instead modulate and fine-tune expression levels of key regulatory proteins. Furthermore, miRNAs allow for more consistent protein expression levels between cells and species by limiting transcriptional noise, and this is hypothesized to have allowed for more complex phenotypes to evolve. [41-43] The repression results from miRNA binding to the complementary sequence of the 3′UTR of the target mRNA.[44] The roles of miRNAs are profound in various physiologic and pathologic processes, such as cellular growth, development, organogenesis, and apoptosis.[45] MiRNAs have been implicated in various disease states that include cancer and cardiovascular diseases, and are considered important therapeutic targets. In addition, miRNAs are involved in most types of inflammatory responses, and there is increasing evidence that miRNAs are effective in regulating the magnitude of inflammatory responses through their effects on cellular development and acute cellular function.[46] Despite the growing importance of miRNAs in pathogenic states, few studies have explored the role of miRNAs in intra-amniotic inflammation and their possible involvement in preterm birth.

A recent microarray analysis compared the miRNAomes in plasma between patients with spontaneous preterm birth and those with delivery at term. A total of eight miRNAs were differentially expressed in preterm birth groups, and

miR-302b, miR-1253, and a cluster of miR-548 miRNAs were significantly under-expressed in preterm birth cases compared to term controls.[47] However, the potential role of these miRNAs in preterm birth remains unknown.

Of the differentially expressed miRNAs in preterm birth groups, the miR-548 cluster and miR-302b were predicted to bind HMGB1 3′UTR by a bioinformatics analysis. Thus, we performed this study to 1) compare the levels of HMGB1 in amniotic fluid of patients with intra-amniotic inflammation to those in healthy controls, 2) compare HMGB1 expression in amnion membrane of patients with preterm birth and chorioamnionitis to normal term controls, and 3) investigate the expression of the miR-548 cluster in amnion membrane and explore the regulation of HMGB1 expression by the miR-548 cluster.

II. MATERIALS AND METHODS

1. Patients and amniotic fluid collection

We investigated amniotic fluid (AF) samples from 48 women with singleton pregnancies and clinically indicated amniocentesis. Samples were obtained by transabdominal amniocentesis for second trimester genetic karyotyping (n=10) or to rule out AF infection in women who had preterm labor contractions refractory to tocolysis or advanced cervical dilatation (n=38). Exclusion criteria were the presence of anhydramnios, human immunodeficiency or hepatitis viral infections, congenital anomalies, or abnormal karyotype. Gestational age was determined based on the last menstrual period confirmed by ultrasound examination prior to 14 weeks gestation. Preterm labor was defined as the presence of regular uterine contractions and documented cervical effacement and/or dilatation in patients before week 37 of gestation. Intra-amniotic inflammation (IAI) was defined by amniotic fluid interleukin (IL)-6 concentrations ≥ 2,600 pg/mL.[48] All women provided written informed consent before the collection of AF and placenta samples. The collection and utilization of the samples were approved by the Ethics Committee of Kangnam Sacred Heart Hospital.

2. Chemical and microbiological studies of AF

Following retrieval under sterile conditions, AF was analyzed for glucose

concentration, white blood cell (WBC) count, gram stain, and for standard culture of aerobic and anaerobic bacteria, including Ureaplasma and Mycoplasma species. The remaining AF was aliquoted into 2-mL vials and cryopreserved at -80^{o}C.

3. Enzyme-linked immunosorbent assay (ELISA) of AF HMGB1 and IL-6

The levels of IL-6 and HMGB1 in AF and cell culture medium (conditioned medium) were determined by an ELISA assay kit (Cusabio, Wuhan, China) according to the manufacturer's instructions. The levels of IL-1ß and IL-8 in culture medium were determined by an ELISA assay kit (Cusabio and RayBiotech, Norcross, GA, USA, respectively) according to the manufacturer's instructions.

4. Amnion collection and processing

Placentas (n=6) were obtained from women with suspected chorioamnionitis caused by prolonged latency after preterm premature rupture of membranes and/or preterm labor (median gestational age of 29.4 weeks, range 25.3-35.0 weeks), and from healthy women (n=4) undergoing elective cesarean delivery at term in the absence of labor (median gestational age 38.1 weeks, range 20.1-39.0 weeks). Amnion membrane was manually stripped from the chorion and divided into small pieces (approximately 1.0 cm^3 each). After sample

collection, the remaining full-thickness placental biopsies were obtained and fixed in formalin. Biopsy specimens were interpreted by a placental pathologist who was blinded to the clinical and laboratory findings. Amnion samples histologically diagnosed with chorioamnionitis were used as patients' samples in the experiments.

5. Isolation of amniotic epithelial cells

Amnion membranes (n=5) were obtained from women who had completed full-term pregnancy during elective cesarean deliveries before the onset of labor and from those (n=3) with preterm birth and chorioamnionitis. The primary human amniotic epithelial cells (hAECs) were isolated from freshly obtained amnion as described previously.[49-52] Briefly, the amnion membrane was washed with ice-cold phosphate buffered saline (PBS, pH 7.2) to remove blood clots and cellular debris, and cut into pieces approximately 6 cm long. The first enzymatic digestion was performed by incubating the membrane pieces with 20 mL of pre-warmed 0.05% trypsin/EDTA (Thermo Fisher Scientific, Waltham, MA, USA) at 37^{o}C for 10 min with gentle shaking. The cells obtained at this step were discarded to remove blood clots and cellular debris. Second and third digestions were conducted the same way. The membrane pieces were transferred into new 50-mL conical tubes containing 20 mL 0.05% trypsin/EDTA and incubated at 37^{o}C for 30 min with gentle shaking. The

digestion mixtures obtained from the second and third digestions were filtered through a 70-μm cell strainer and centrifuged at 500 × g for 10 min. Cell pellets were suspended and grown in DMEM/F12 (Gibco, Franklin Lakes, NJ, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 10 ng/mL epidermal growth factor (R&D Systems, Madison, WI, USA).

6. Cell culture and reagents

The primary hAECs from amnion tissues were plated and maintained in culture at 37°C in a humidified incubator in an atmosphere of 5% CO_2. JetPrime transfection reagent was purchased from PolyPlus-Transfection (Strasbourg, France). Antibodies to HMGB1 (Cell Signaling Technology, Beverly, MA, USA) and Actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. Lipopolysaccharide (LPS) from *Escherichia coli* strain 0111:B4 was obtained from Sigma-Aldrich.

7. Western blot analysis

Western blot analysis was performed according to standard procedures.[37] Tissue and cell lysates were prepared using lysis buffer (62.5 mm Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 50 mm dithiothreitol, 0.01% (w/v) bromphenol blue, 10 mm NaF, 1% (v/v) protease inhibitor mixture, 1 mm

sodium orthovanadate). The samples were boiled for 5 min, and equal amounts of protein (20 μg/well) were analyzed on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred onto a polyvinylidene fluoride membranes (Thermo Fisher Scientific) and subjected to immunoblotting. The dilution of each primary antibody was empirically determined. After extensive washing, blots were further incubated with an anti-mouse IgG-horseradish peroxidase-conjugated antibody at a 1:3,000 dilution for 1 h at room temperature and were developed using an enhanced chemiluminescence reagents (Thermo Fisher Scientific).

8. MicroRNA prediction of target genes and functional and bioinformatics analysis

Binding sites and sequences of miR-548 cluster on the HMGB1 3'UTR were predicted by the target prediction programs TargetScan (http://www.targetscan.org), MiRDB (http://mirdb.org), and miRmap (https://mirmap.ezlab.org). Paired sequence alignment is marked by red color and continuous lines (Table 1).

Table 1. Predicted sequence alignment between miRNAs and HMGB1 3'UTR

ID		Alignment	Position
miR-548aa	target (5'→3')	UUUAGGGAACAAUUUGGCAAUUUUGUGGUUUUCGAGAUUAU	1298-1305
	miRNA (3'→5')	ACCACGUUUUCAUUAACACCAAAAA	
	target (5'→3')	AUAAAUAGGUUAAUGGGCUGAAAAGGUUUUGUCAAACAU	1681-1686
	miRNA (3'→5')	ACCACGUUUUCAUUAACACCAAAAA	
	target (5'→3')	GAUACUAAUAAACUAAUAAUUGCAGAGGUUUUAAAUGCUAG	1499-1504
	miRNA (3'→5')	ACCACGUUUUCAUUAACACCAAAAA	
miR-548ai	target (5'→3')	UUUCUAAUAAUACCUCUUAGCAGUACCUUUUAAAAUAAG	1591-1596
	miRNA (3'→5')	CCCUUUUUGACGUUAAUGGAAA	
miR-548a-3p	target (5'→3')	AAUUAGUUAUAUAUGGGGAUGGUAGUUUUUUCAUCUUC	306-311
	miRNA (3'→5')	CGUUUUCAUUAACGGUCAAAAC	
	target (5'→3')	AUGAACUAAGAUGUAUAAAUCUUAGUUUUUUUUGUAUUU	2376-2381
	miRNA (3'→5')	CGUUUUCAUUAACGGUCAAAAC	
	target (5'→3')	UUUAAGAUGUUGAGAAUGGGAACAGUUUUUUUAGGGUU	2024-2030
	miRNA (3'→5')	CGUUUUCAUUAACGGUCAAAAC	
miR-548x-5p	target (5'→3')	CUUGAGGGGAAGCUAGUCUUUUGCUUUUGCCCAUUUUGA	523-529
	miRNA (3'→5')	GUUUUUGACGUUAAUGAAAACGU	
	target (5'→3')	UUUCUUCUUGAGGGGAAGCUAGUCUUUUGCUUUUGCCCA	516-523
	miRNA (3'→5')	GUUUUUGACGUUAAUGAAAACGU	
	target (5'→3')	CAUUACCAUGUAAUGGCAGUUAUAUUUUGCAGUUCCCAC	869-874
	miRNA (3'→5')	GUUUUUGACGUUAAUGAAAACGU	

9. RNA isolation and quantitative PCR (qPCR)

Total RNA samples were isolated from cells and amnion tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Five micrograms of RNA was used for cDNA synthesis using the Maxime RT PreMix Kit (iNtRON Biotechnology, Seoul, South Korea). PCR reactions were performed using a 2× Rotor-Gene SYBR Green PCR Master Mix (Qiagen, Carlsbad, CA, USA) in the Rotor-Gene Q (Qiagen). The primers used were HMGB1 (forward): 5′-ACATCCAAAATCTTGATCAGTTA-3′ and (reverse) -3′ (reverse) 5′-CTCCTTAATGTC ACGCACGA-3′; and Actin (forward): 5′-CATGTACGTTGCTA TCCAGGC-3′ (reverse) 5′-CTCCTTAATGTCACGCACGA-3′. For the analysis of miRNA expression, miRNAs were isolated from the hAECs and amnion tissues using miRNeasy (Qiagen), followed by reverse transcription with the miScript Transcription Kit (Qiagen). The miRNA expression level was measured with a miScript SYBR Green PCR Kit (Qiagen) using the Rotor-Gene Q (Qiagen). Primers for miRNAs and endogenous control U6 gene are shown in Tables 2 and 3.

Table 2. Sequences of the primers used in real-time RT-PCR

Designation	Sequence (5'→3')
miR-548aa	AAAAACCACAATTACTTTTGCACCA
miR-548ai	AAAGGTAATTGCAGTTTTTCCC
miR-548a-3p	CAAAACTGGCAATTACTTTTGC
miR-548x-5p	TGCAAAAGTAATTGCAGTTTTTG
U6	CTCGCTTCGGCAGCACA

Table 3. Sequences of miR-mimic, miR-inhibitor, and controls

Mimics or inhibitors	Sequence (5'→3')	
Mimics		
control mimic	sense	UUCUCCGAACGUGUCACGUTT
	antisense	ACGUGACACGUUCGGAGAATT
miR-548aa mimic	sense	AAAAACCACAAUUACUUUUGCACCA
	antisense	GUGCAAAAGUAAUUGUGGUUUUUUU
miR-548ai mimic	sense	AAAGGUAAUUGCAGUUUUUCCC
	antisense	GAAAAACUGCAAUUACCUUUUU
miR-548a-3p mimic	sense	CAAAACUGGCAAUUACUUUUGC
	antisense	AAAAGUAAUUGCGAGUUUUACC
miR-548x-5p mimic	sense	UGCAAAAGUAAUUGCAGUUUUUG
	antisense	UAAAAACUGCAAUUACUUUC
Inhibitors		
miR-control inhibitor	CAGUACUUUUGUGUAGUACAA	
miR-548aa inhibitor	UGGUGCAAAAGUAAUUGUGGUUUUU	
miR-548ai inhibitor	GGGAAAAACUGCAAUUACCUUU	
miR-548a-3p inhibitor	GCAAAAGUAAUUGCCAGUUUUG	
miR-548x-5p inhibitor	CAAAAACUGCAAUUACUUUUGCA	

10. Forced and repressed expression of miRNA-548 cluster

To verify the effect of miRNAs, miR-548 inhibitor, mimic, and negative control groups were transfected using JetPrime (Polyplus-Transfection). Total RNA, including miRNA and proteins, were isolated at 36 h post-transfection and were used for western blot analysis and qPCR. The sequences of miR-inhibitor and mimic are listed in Table 2. hAECs were incubated in the presence of 10 and 100 ng/mL LPS to induce inflammation. Culture medium was collected after 24 h of incubation to measure the levels of HMGB1.

11. Statistical analyses

All experiments were repeated at least three separate times. Statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Data were compared with Student's *t* test for parametric data or Mann-Whitney *U* test for nonparametric data. Spearman correlations were used to measure co-linearity between the selected independent variables. Comparisons between proportions were done with Fisher's exact test. Statistical analysis of the immunoassays data was performed after logarithmic transformation of data. Statistical significance was indicated when $p<0.05$.

III. RESULTS

1. HMGB1 expression in AF and amnion membrane

We analyzed HMGB1 levels in AF obtained from women with signs or symptoms of preterm birth who had amniocentesis to rule out infection (n=38) or for either genetic indication (n=10). Table 4 shows a comparison of clinical characteristics of patients with amniocentesis. Thirty four of 48 patients were diagnosed with intra-amniotic inflammation. The median [interquartile range] AF concentration of HMGB1 was significantly higher in patients with intra-amniotic inflammation than in those without intra-amniotic inflammation (563.8 [373.0-800.0] vs 273.0 [47.0-539.3] pg/mL, p=0.004; Figure 1, A). The levels of HMGB1 significantly correlated with the levels of IL-6 (r=0.571; p<0.001; Figure 1, C).

Table 4. Clinical characteristics of patients with amniocentesis

	Patients with IAI (n=34)	Normal controls (n=14)	p-value
Maternal age (years)	33 [30.8-36.0]	36.5 [33.8-38.5]	0.005
Previous preterm delivery	5 (14.7)	1 (7.1)	0.656
GA at amniocentesis (weeks)	21 [19.6-22.4]	17.4 [16.7-19.9]	<0.001
GA at delivery (weeks)	29.4 [21.0-38.0]	38.0 [37.0-38.5]	0.002
Maternal WBC count (cell/mL)	9,750 [8,375-12,350]	8,885 [7,395-11,012]	0.353
Amniotic fluid analysis			
HMGB1 (pg/mL)	563.8 [373.0-800.0]	273.0 [47.0-539.3]	0.004
IL-6 (pg/mL)	6,022 [5,199-6,224]	588 [465-1,175]	<0.001
Glucose < 20 mg/dL, n (%)	10 (29.4)	0	0.023
WBC (cells/mm^3)	40 [7.0-137.5]	7.0 [2.0-9.3]	0.019
Positive cultures	2 (5.9)	0	1.000

Data presented as median [interquartile range] or n (%).

GA, gestataional age; IAI, intra-amniotic inflammation; WBC, white blood cell; HMGB1, High mobility group box 1; IL-6, interleukin-6

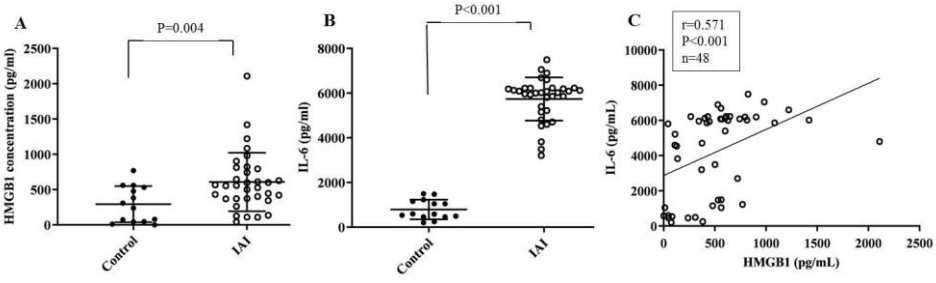

Figure 1. Levels of HMGB1 and IL-6 in amniotic fluid. A, B, Levels of HMGB1 and IL-6 in amniotic fluid of women with and without intra-amniotic inflammation. C, Relationship of amniotic fluid HMGB1 to the acute phase cytokine interleukin (IL)-6.

The expressions of HMGB1 mRNA and protein were increased in amnion membranes from women with preterm birth and chorioamnionitis as compared to normal controls (Figure 2, A). To confirm these data, we measured the expression level of HMGB1 in primary human amnion epithelial cells isolated from amnion membrane obtained from patients who delivered preterm due to chorioamnionitis (patient-derived hAECs, PD-hAECs) and women who had full-term pregnancy as normal control (hAECs). As expected, HMGB1 expression in PD-hAECs was significantly higher than in hAECs (Figure 2, B). Additionally, HMGB1 release from PD-hAECs was highly augmented compared to hAECs (Figure 2, C). These date implicated HMGB1 as a novel candidate biomarker for the early prediction of preterm birth determined by chorioamnionitis.

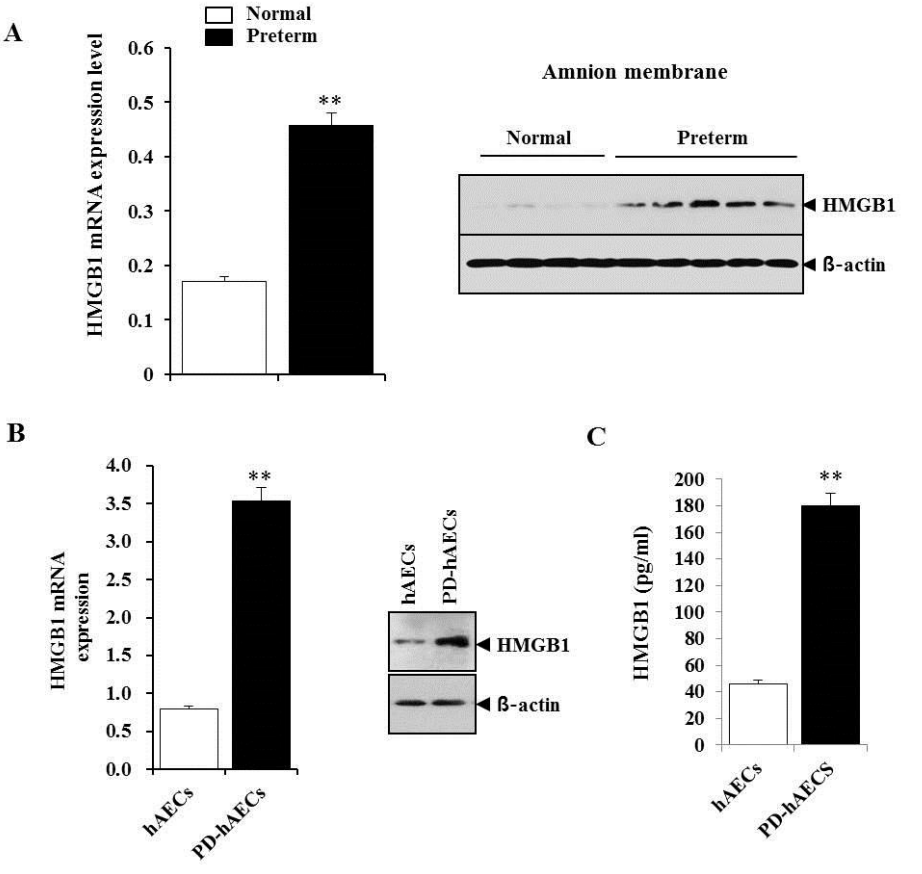

Figure 2. HMGB1 mRNA and protein expression in amnion membranes and human amniotic epithelial cells (hAECs). A, HMGB1 mRNA and protein expression in the amnion membrane from women with preterm birth and chorioamnionitis and from normal term controls. B, HMGB1 mRNA and protein expression in hAECs from patients with preterm birth and chorioamnionitis and from normal term controls. C, HMGB1 levels in culture medium of hAECs from patients with preterm birth and chorioamnionitis and from normal term controls. **significant difference ($P<0.001$) compared with control group. hAECs, human amniotic epithelial cells; PD-hAECs, patient-derived human amniotic epithelial cells.

2. MiR-548 cluster is under-expressed in amnion membrane from patients with preterm birth and chorioamnionitis

Several miRNAs including the miR-548 cluster are reportedly downregulated in spontaneous preterm birth cases compared to controls.[15] Thus, we investigated the correlation between HMGB1 and the miR-548 cluster. Based on the computational prediction program, several binding sites of the miR-548 cluster were predicted in the 3'UTR of HMGB1 (Table 1). To explore whether the miR-548 cluster could be a negative regulator of HMGB1 in preterm birth with chorioamnionitis, the expression level of the miR-548 cluster including miR-548aa, miR-548ai, miR-548-3p, and miR-548x-5p was measured using qPCR in the amnion membrane. Also, we investigated miR-302b, which displays reduced expression in spontaneous preterm birth.[15] As shown in Figure 3, A, all members of the miR-548 cluster were significantly decreased in amnion membranes from preterm women with chorioamnionitis compared to those from normal term controls. Furthermore, the expression of the miR-548 cluster was reduced in PD-hAECs than in hAECs from normal healthy women (Figure 3, B). However, there was no significant difference in the expression level of miR-302b between preterm and normal controls (data not shown). These results suggested that miR-548 cluster could be an inhibitory regulator of HMGB1 expression in preterm birth with chorioamnionitis.

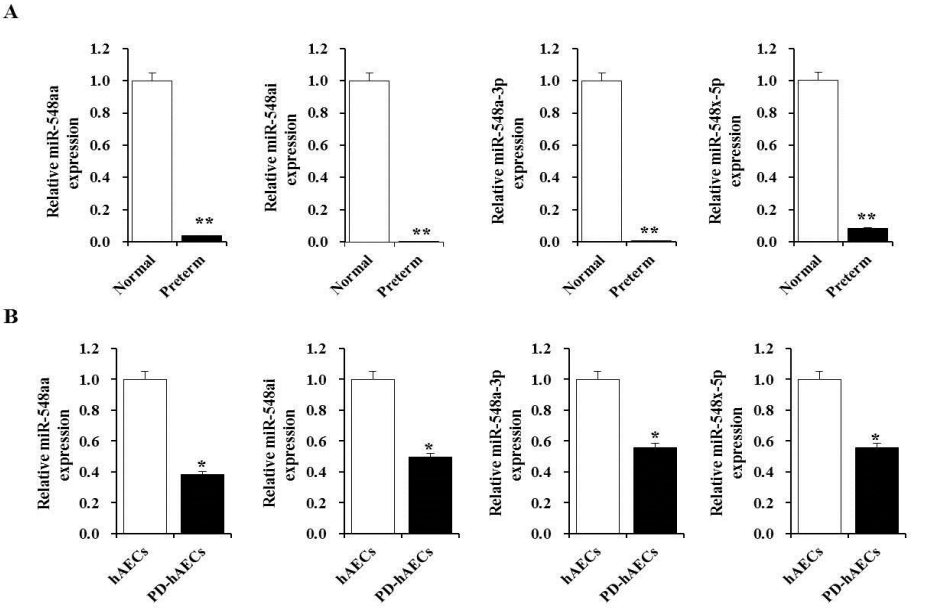

Figure 3. MicroRNA-548 cluster expression in amnion membranes and human amniotic epithelial cells (hAECs). A, Levels of miRNA-548aa, miRNA-548ai, miRNA-548a-3p, and miRNA-548x-5p in amnion membranes from women with preterm birth and chorioamnionitis and from normal term controls. B, Levels of miRNA-548aa, miRNA-548ai, miRNA-548a-3p, and miRNA-548x-5p in patient-derived hAECs (PD-hAECs) and hAECs from normal healthy women. **significant difference ($P<0.01$) compared with control group, *significant difference ($P<0.05$) compared with control group. hAECs, human amniotic epithelial cells.

3. *MiR-548 cluster can regulate expression of HMGB1 in human amniotic epithelial cells (hAECs)*

To validate the correlation between HMGB1 and the miR-548 cluster in patients with preterm birth and chorioamnionitis, we investigated whether the expression level of miR-548 cluster is crucial to the HMGB1 expression level using the synthetic mimics and inhibitors for miR-548 cluster (Table 3) in hAECs. To examine whether downregulation of miR-548 cluster could induce the enhancement of HMGB1 expression, hAECs were transfected with miR-548aa inhibitor, miR-548ai inhibitor, miR-548a-3p inhibitor, or miR-548x-5p inhibitor followed by qPCR (Figure 4, A). Inhibitors of the miR-548 cluster obviously increased the expression of both mRNA and protein levels of HMGB1 in hAECs compared to control inhibitor (Figure 4, B). Moreover, HMGB1 levels increased in the culture medium of hAECs transfected with miR-548 inhibitor compared to those transfected with control inhibitor (Figure 4, C). These findings indicated that downregulation of the miR-548 cluster contributed to the overexpression of HMGB1 expression in preterm birth with chorioamnionitis. In addition, the negative correlation between the expression of the miR-548 cluster and HMGB1 level was implicated in preterm birth with chorioamnionitis.

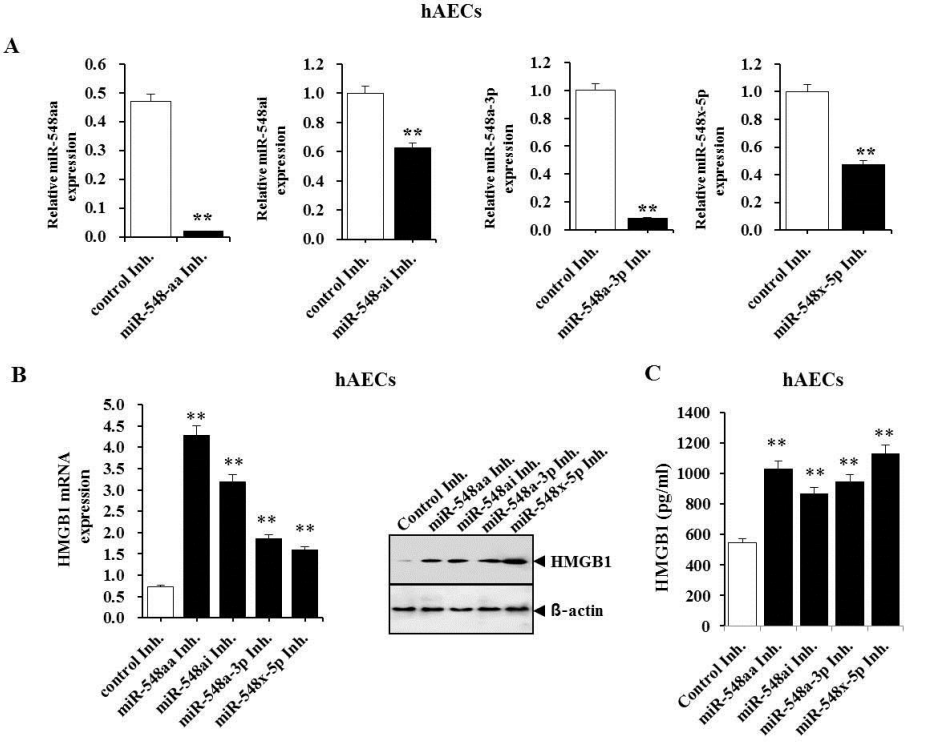

Figure 4. MicroRNA-548 cluster can regulate expression of HMBG1 in human amniotic epithelial cells (hAECs). A, Levels of miRNA-548aa, miRNA-548ai, miRNA-548a-3p, and miRNA-548x-5p in hAECs transfected with miRNA-548aa inhibitor, miRNA-548ai inhibitor, miRNA-548a-3p inhibitor, miRNA-548x-5p inhibitor, and control inhibitor. **B**, HMGB1 mRNA and protein expression in hAECs transfected with miRNA-548 inhibitor and with control inhibitor. **C**, HMGB1 levels in culture medium of hAECs transfected with miRNA-548 inhibitor and with control inhibitor. **significant difference ($P<0.01$) compared with control group. hAECs, human amniotic epithelial cells.

4. LPS-induced inflammation induces enhanced HMGB1 expression through downregulation of miR-548 cluster

To evaluate the role of the negative correlation between the miR-548 cluster and HMGB1 in preterm birth with chorioamnionitis, hAECs were treated with LPS (10 or 100 ng/mL) for 24 h to induce inflammation followed by measurement of the expression levels of the miR-548 cluster and HMGB1. Compared to control, LPS inhibited the expression of the miR-548 cluster and led to increases of HMGB1 mRNA and protein in hAECs (Figure 5, A and B). A significant elevation of HMGB1 levels was also observed in the culture medium of LPS-stimulated hACEs as compared to normal control medium (Figure 5, C). These findings indicated that downregulation of the miR-548 cluster promoted HMGB1 expression in hAECs and its subsequent release from the cells upon LPS-mediated inflammation.

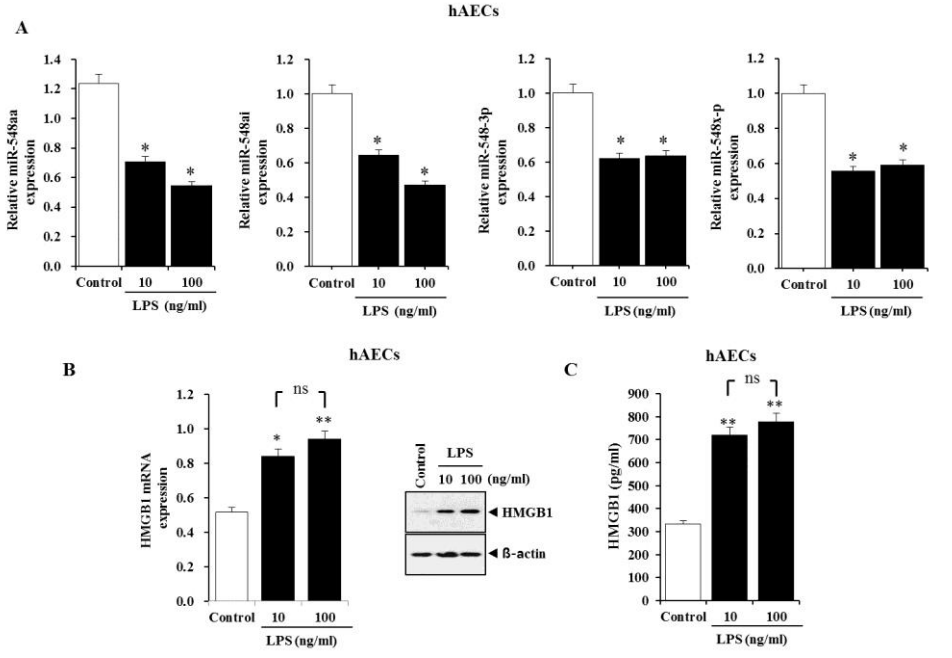

Figure 5. Lipopolysaccharide (LPS)-mediated inflammation induces enhanced HMGB1 expression through downregulation of microRNA-548 cluster. A, Levels of miRNA-548aa, miRNA-548ai, miRNA-548a-3p, and miRNA-548x-5p in hAECs treated with 10 or 100 ng/mL LPS for 24 h. **B**, HMGB1 mRNA and protein expression in hAECs treated with 10 or 100 ng/mL LPS for 24 h. **C**, HMGB1 levels in culture medium of hAECs treated with 10 or 100 ng/mL LPS for 24 h. **significant difference ($P<0.01$) compared with control group, *significant difference ($P<0.05$) compared with control group. hAECs, human amniotic epithelial cells; LPS, Lipopolysaccharide.

5. MiR-548 cluster could attenuate the LPS-induced inflammation in hAECs

To verify the effect of the miR-548 cluster on LPS-induced expression and release of HMGB1, hAECs were transfected with miR-548aa mimic, miR-548ai mimic, miR-548a-3p mimic, miR-548x-5p mimic, or control mimic. HMGB1 mRNA and protein expressions were pronounced in hAECs transfected with control mimic stimulated by LPS. In contrast, overexpression of the miR-548 cluster in cells transfected with miR-548 cluster mimics lowered HMGB1 induction upon LPS exposure (Figure 6, A). Moreover, HMGB1 levels were decreased in the culture medium of the miR-548 mimic transfected hAECs as compared to control mimic cells (Figure 6, B). Those findings indicated that the miR-548 mimic inhibited HMGB1 up-regulation in hAECs and the subsequent release of HMGB1 from hAECs exposed to an inflammatory stimulus. Furthermore, release of inflammatory cytokines (IL-1ß and IL-6) and chemokine (IL-8) was significantly reduced in the culture medium of hAECs transfected with miR-548 cluster mimics compared to control mimic cells. The data indicated that the overexpression of miR-548 cluster can suppress the inflammatory responses in hAECs.

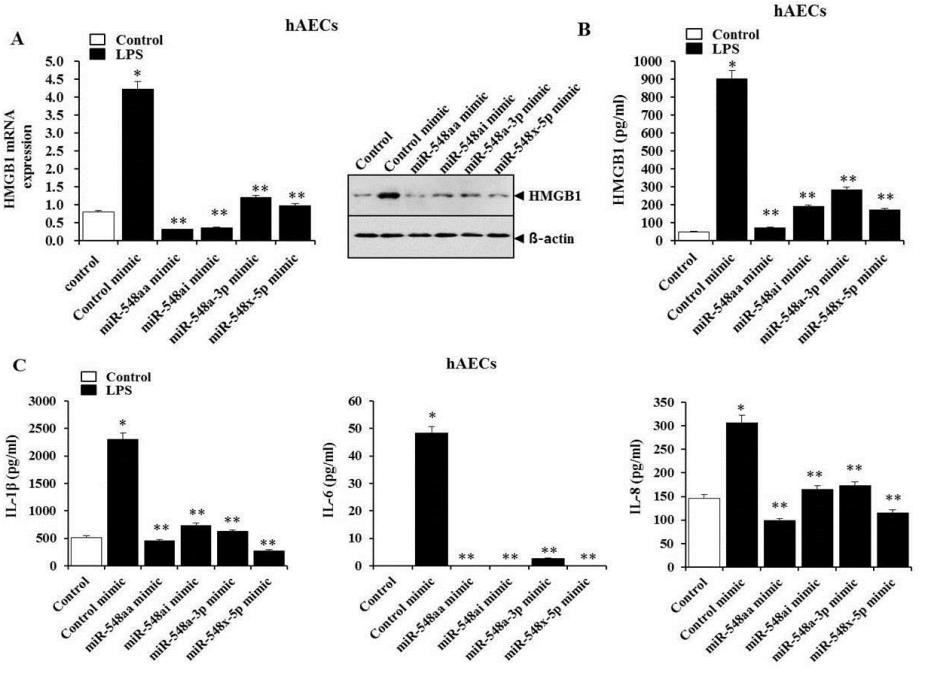

Figure 6. MicroRNA-548 cluster can attenuate lipopolysaccharide (LPS)-induced inflammation in hAECs. A, HMGB1 mRNA and protein expression in miRNA-548 mimic and control mimic transfected hAECs that were not treated or treated with LPS. **B**, HMGB1 levels in culture medium of miRNA-548 mimic and control mimic transfected hAECs that were not treated or treated with LPS. **C**, IL-1β , IL-6, and IL-8 levels in culture medium of miRNA-548 mimic and control mimic transfected hAECs that were not treated or treated with LPS. **significant difference ($P<0.001$) compared with control mimic group, *significant difference ($P<0.001$) compared with control group. hAECs, human amniotic epithelial cells; LPS, Lipopolysaccharide; IL, interleukin.

IV. DISCUSSION

In this study, we confirmed that the HMGB1 levels in AF from the patients with IAI were significantly higher than from those without IAI, and that HMGB1 was highly expressed in amnion membranes of patients with preterm birth and chorioamnionitis as compared to those in normal term controls. In addition, we validated that LPS-mediated inflammation could be responsible of the observed HMGB1 up-regulation in hAECs and for the release of HMGB1 from hAECs *in vitro*. Our findings, confirmed both *in vivo* and *in vitro,* were consistent with the fact the inflammation induced the expression of HMGB1 in the amnion membrane and its release from the amnion epithelial cells. HMGB1 release actively occurs when monocytes, macrophages, natural killer cells, dendritic cells, endothelial cells, and other immunologically competent cells are exposed to microbe-associated molecular patterns, pathogen-associated molecular patterns, and endogenously derived inflammatory mediators, including tumor necrosis factor, IL-1, and interferon-gamma. Neurons, astrocytes, erythroleukemia cells, neuroblastoma cells, and other tumor cells are also reportedly able to actively secrete HMGB1.[25,27,28,30,53] Passive release of HMGB1 can be initiated when cellular integrity is damaged.[37] Considering that HMGB1 expression in hAECs increased when the cells were stimulated by LPS, HMGB1 could be released actively from amnion epithelial cells. Moreover, inflammation of the amnion membrane might lead to injury of amnion epithelial cells, which might cause the passive release of HMGB1. Regardless of the

mechanism, the elevated levels of HMGB1 in AF could be considered a marker for amnion epithelial cell damage and injury, and representative of active signal transduction in amnion epithelial cells. It remains unclear how the released HMGB1 affects the amnion epithelial cells and plays a role in leading to preterm birth. We speculate that HMGB1 might induce cytokine, chemokine, and metalloproteinase synthesis that might be involved in preterm birth.

Significant suppression of the miR-548 cluster was observed in the amnion membrane from patients with preterm birth and chorioamnionitis compared to normal term controls. Moreover, when hAECs were treated with LPS, miR-548 was downregulated. A previous study reported that inflammation does alter miRNAs at intestinal epithelial barriers and regulates intestinal permeability by degradation of tight junction protein, which is the target protein of miRNAs.[54] Furthermore, in cervical cells, inflammation can significantly alter the expression of specific miRNAs; these alterations might be involved in cervical remodeling and preterm birth.[55] Indeed, we found that inflammation induced miR-548 downregulation in the amnion membrane and the suppression of miR-548 was associated with preterm birth and chorioamnionitis.

Little is known about the involvement of the miR-548 family in disease processes. Downstream targets of the miR-548 family are involved in breast cancer, inflammatory responses, and potential estrogen receptor sensitivity, indicating a potential biological role in the setting of preterm birth.[56-58]

Furthermore, miR-548 associated target genes have been implicated in breast cancer and immune response.[56,58] A recent study reported the suppression of endogenous miR-548 levels in the setting of viral infections.[58] Similarly, we observed that downregulation, rather than up-regulation, of miR-548 seemed to be important in the etiology of disease. Although the mechanism of miR-548 downregulation following inflammatory stimuli has not been clarified, our results suggest that under-expressed miR-548 can play an important role in chorioamnionitis determined preterm birth.

The inhibition of miR-548 induced the up-regulation of HMGB1 mRNA and protein expression, and release of HMGB1 in the culture medium of hAECs transfected with miR-548 cluster inhibitors. Four miRNAs (miR-548 aa, miR-548ai, miR-548a-3p, and miR-548x-5p) were computationally predicted to bind HMGB1 3′UTR. The HMGB1 levels were inversely correlated with the miR-548 cluster expression in patients with preterm birth and chorioamnionitis. Additionally, LPS downregulated the expression of the miR-548 cluster in hAECs. These findings suggest that IAI can induce downregulation of the miR-548 cluster in the amnion membrane, thus leading to the up-regulated expression of HMGB1 in amnion and its release from amnion epithelial cells. Moreover, the miR-548 cluster mimic could reverse the LPS-induced up-regulation of HMGB1 in hAECs compared to control mimic. The release of HMGB1 from hAECs was also significantly decreased in hAECs transfected

with miR-548 cluster mimics. In addition, the release of inflammatory cytokines and chemokine was suppressed in the culture medium of hAECs transfected with miR-548 cluster mimics compared to control mimic cells. These findings suggest that miR-548 cluster mimics can inhibit the up-regulation of HMGB1 that occurs when inflammation is stimulated, and alters inflammatory responses in hAECs. Thus, we can speculate that miR-548 could effectively reverse and prevent the activation of innate immunity, and significantly attenuate damage in IAI. Although we did not demonstrate the direct binding of the miR-548 cluster to HMGB1, our results suggest that miR-548 regulates IAI by regulating HMGB1, which may be developed as a potential candidate treatment of IAI determined preterm birth.

V. CONCLUSION

Intra-amniotic inflammation induces the up-regulation of HMGB1 expression in the amnion membrane and HMGB1 release via suppression of miR-548. Moreover, miR-548 can attenuate inflammatory responses in amnion epithelial cells. Although the specific regulatory mechanism of miR-548 on HMGB1 expression remains unclear, these data demonstrate a potential molecular mechanism underlying the inhibitory effect of miR-548 on preterm birth, and implicate miR-548 as a potential therapeutic target in preterm birth with chorioamnionitis.

REFERENCES

1. Mazaki-Tovi S, Romero R, Kusanovic JP, et al.: Recurrent preterm birth. Semin Perinatol 2007;31:142-158.

2. Joseph KS, Liu S, Rouleau J, et al.: Influence of definition based versus pragmatic birth registration on international comparisons of perinatal and infant mortality: population based retrospective study. BMJ. 2012;344:e746.

3. Liu L, Johnson HL, Cousens S, et al.: Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. Lancet. 2012;379 (9832):2151-61.

4. Lockwood CJ, Kuczynski E: Risk stratification and pathological mechanisms in preterm delivery. Paediatr Perinat Epidemiol. 2001;15 (Suppl 2):78-89.

5. Romero R, Gomez R, Mazor M, et al.: The preterm labor syndrome. Elder MG Romero R Lamont RF Preterm labor. 1997 Churchill Livingstone New York 29-49

6. Romero R, Avila C, Sepulveda W, et al.: The role of systemic and intrauterine infection in preterm labor. Fuchs A Fuchs F Stubblefield P Preterm birth: causes, prevention, and management. 1993 McGraw-Hill New York

7. Smith R: Parturition. N Engl J Med. 2007;356 (3):271-83.

8. Bennett PR, Elder MG, Myatt L: The effects of lipoxygenase metabolites of arachidonic acid on human myometrial contractility. Prostaglandins. 1987;33:837-44.

9. Ellwood DA, Mitchell MD, Anderson AB, et al.: The in vitro production of prostanoids by the human cervix during pregnancy: preliminary observations. Br J Obstet Gynaecol. 1980;87:210-4.

10. MacDonald PC, Schultz FM, Duenhoelter JH, et al.: Initiation of human parturition. I: Mechanism of action of arachidonic acid. Obstet Gynecol. 1980;44:629-36.

11. Elovitz MA, Mrinalini C: Animal models of preterm birth. Trends Endocrinol Metab. 2004;15:479-87.

12. Fidel PL Jr, Romero R, Wolf N, et al.: Systemic and local cytokine profiles in endotoxin-induced preterm parturition in mice. Am J Obstet Gynecol. 1994;170:1467-75.

13. Gravett MG, Witkin SS, Haluska GJ, et al.: An experimental model for intraamniotic infection and preterm labor in rhesus monkeys. Am J Obstet Gynecol. 1994;171:1660-7.

14. Romero R, Salafia CM, Athanassiadis AP, et al. The relationship between acute inflammatory lesions of the preterm placenta and amniotic fluid microbiology. Am J Obstet Gynecol 1992;166:1382-8.

15. Yoon BH, Jun JK, Romero R, et al. Amniotic fluid inflammatory cytokines (interleukin-6, interleukin-1beta, and tumor necrosis factor-alpha), neonatal brain white matter lesions, and cerebral palsy. Am J Obstet Gynecol 1997;177:19-26.

16. Romero R, Espinoza J, Goncalves LF, Kusanovic JP, Friel L, Hassan S. The role of inflammation and infection in preterm birth. Semin Reprod Med 2007;25:21-39.

17. Christiaens I, Zaragoza DB, Guilbert L, Robertson SA, Mitchell BF, Olson DM. Inflammatory processes in preterm and term parturition. J Reprod Immunol 2008;79:50-7.

18. Vrachnis N, Vitoratos N, Iliodromiti Z, Sifakis S, Deligeoroglou E, Creatsas G. Intrauterine inflammation and preterm delivery. Ann N Y Acad Sci 2010;1205:118-22.

19. Friel LA, Romero R, Edwin S, et al. The calcium binding protein, S100B, is increased in the amniotic fluid of women with intra-amniotic infection/inflammation and preterm labor with intact or ruptured membranes. J Perinat Med 2007;35:385-93.

20. Chaiworapongsa T, Erez O, Kusanovic JP, et al. Amniotic fluid heat shock protein 70 concentration in histologic chorioamnionitis, term and preterm parturition. J Matern Fetal Neonatal Med 2008;21:449-61.

21. Romero R, Durum S, Dinarello CA, Oyarzun E, Hobbins JC, Mitchell MD. Interleukin-1 stimulates prostaglandin biosynthesis by human amnion. Prostaglandins 1989;37:13-22.

22. Romero R, Mazor M, Brandt F, et al. Interleukin-1 alpha and interleukin-1 beta in preterm and term human parturition. Am J Reprod Immunol 1992;27:117-23.

23. Romero R, Chaiworapongsa T, Alpay Savasan Z, et al. Damage-associated molecular patterns (DAMPs) in preterm labor with intact membranes and preterm PROM: a study of the alarmin HMGB1. J Matern Fetal Neonatal Med 2011;24:1444-55.

24. Romero R, Chaiworapongsa T, Savasan ZA, et al. Clinical chorioamnionitis is characterized by changes in the expression of the alarmin HMGB1 and one of its receptors, sRAGE. J Matern Fetal Neonatal Med 2012;25:558-67.

25. Andersson U, Tracey KJ. HMGB1 is a therapeutic target for sterile inflammation and infection. Annu Rev Immunol 2011;29:139-62.

26. Buhimschi IA, Zhao G, Pettker CM, et al. The receptor for advanced glycation end products (RAGE) system in women with intraamniotic infection and inflammation. Am J Obstet Gynecol 2007;196:181.e1-13.

27. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 2002;418:191-5.

28. Rovere-Querini P, Capobianco A, Scaffidi P, et al. HMGB1 is an endogenous immune adjuvant released by necrotic cells. EMBO Rep 2004;5:825-30.

29. Chen G, Ward MF, Sama AE, Wang H. Extracellular HMGB1 as a proinflammatory cytokine. J Interferon Cytokine Res 2004;24:329-33.

30. Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol 2005;5:331-42.

31. Dumitriu IE, Baruah P, Manfredi AA, Bianchi ME, Rovere-Querini P. HMGB1: guiding immunity from within. Trends Immunol 2005;26:381-7.

32. Bianchi ME, Manfredi AA. High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. Immunol Rev 2007;220:35-46.

33. Klune JR, Dhupar R, Cardinal J, Billiar TR, Tsung A. HMGB1: endogenous danger signaling. Mol Med 2008;14:476-84.

34. Castiglioni A, Canti V, Rovere-Querini P, Manfredi AA. High-mobility group box 1 (HMGB1) as a master regulator of innate immunity. Cell Tissue Res 2011;343:189-99.

35. Harris HE, Andersson U, Pisetsky DS. HMGB1: a multifunctional alarmin driving autoimmune and inflammatory disease. Nat Rev Rheumatol 2012;8:195-202.

36. Diener KR, Al-Dasooqi N, Lousberg EL, Hayball JD. The multifunctional alarmin HMGB1 with roles in the pathophysiology of sepsis and cancer. Immunol Cell Biol 2013;91:443-50.

37. Gauley J, Pisetsky DS. The translocation of HMGB1 during cell activation and cell death. Autoimmunity 2009;42:299-301.

38. Baumbusch MA, Buhimschi CS, Oliver EA, et al. High Mobility Group-Box 1 (HMGB1) levels are increased in amniotic fluid of women with intra-amniotic inflammation-determined preterm birth, and the source may be the damaged fetal membranes. Cytokine 2016;81:82-7.

39. Gomez-Lopez N, Romero R, Plazyo O, et al. Intra-Amniotic Administration of HMGB1 Induces Spontaneous Preterm Labor and Birth. Am J Reprod Immunol 2016;75:3-7.

40. Menon R. Human fetal membranes at term: Dead tissue or signalers of

parturition? Placenta 2016;44:1-5.

41. Bartel DP, Chen CZ. Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nat. Rev. Genet. 2004; 5:396–400.

42. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat. Rev. Cancer 2006;6:857–66.

43. CalinGA, DumitruCD, ShimizuM, BichiR, ZupoS, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc. Natl. Acad. Sci. USA 2002; 99:15524–29.

44. Ambros V. The functions of animal microRNAs. Nature 2004;431:350-5.

45. Sonkoly E, Pivarcsi A. microRNAs in inflammation. Int Rev Immunol 2009;28:535-61.

46. Gray C, McCowan LM, Patel R, Taylor RS. Maternal plasma miRNAs as biomarkers during mid-pregnancy to predict later spontaneous preterm birth: a pilot study. 2017;7:815.

47. Romero R, Miranda J, Chaiworapongsa T, et al. Prevalence and clinical significance of sterile intra-amniotic inflammation in patients with preterm labor and intact membranes. Am J Reprod Immunol 2014;72:458-74.

48. Behnia F, Taylor BD, Woodson M, et al. Chorioamniotic membrane senescence: a signal for parturition? Am J Obstet Gynecol 2015;213:359.e1-16.

49. Menon R, Boldogh I, Hawkins HK, et al. Histological evidence of oxidative stress and premature senescence in preterm premature rupture of the human fetal membranes recapitulated in vitro. Am J Pathol 2014;184:1740-51.

50. Menon R, Boldogh I, Urrabaz-Garza R, et al. Senescence of primary amniotic cells via oxidative DNA damage. PLoS One 2013;8:e83416.

51. Motedayyen H, Esmaeil N, Tajik N, et al. Method and key points for isolation of human amniotic epithelial cells with high yield, viability and purity. BMC Res Notes 2017;10:552.

52. Kim Y, Park H, Park D, et al. Cancer/testis antigen CAGE exerts negative regulation on p53 expression through HDAC2 and confers resistance to anti-cancer drugs. J Biol Chem 2010;285:25957-68.

53. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol 2007;81:1-5.

54. Ye D, Guo S, Al-Sadi R, Ma TY. MicroRNA regulation of intestinal epithelial tight junction permeability. Gastroenterology 2011;141:1323-33.

55. Elovitz MA, Brown AG, Anton L, Gilstrop M, Heiser L, Bastek J. Distinct cervical microRNA profiles are present in women destined to have a preterm birth. Am J Obstet Gynecol 2014;210:221.e1-11.

56. Shi Y, Qiu M, Wu Y, Hai L. MiR-548-3p functions as an anti-oncogenic regulator in breast cancer. Biomed Pharmacother 2015;75:111-6.

57. Elovitz MA, Anton L, Bastek J, Brown AG. Can microRNA profiling in maternal blood identify women at risk for preterm birth? Am J Obstet Gynecol 2015;212:782.e1-5.

58. Sanders AP, Burris HH, Just AC, et al. microRNA expression in the cervix during pregnancy is associated with length of gestation. Epigenetics 2015;10:221-8.

ABSTRACT(IN KOREAN)

융모양막염이 있는 조산환자에서 microRNA-548에 의한 high mobility group box 1 발현 조절에 대한 연구

<지도교수 김영한>

연세대학교 대학원 의학과

손가현

자궁내감염 및 염증은 융모양막염에서 흔히 동반되며 이는 자연조산 및 불량한 임신 예후와 연관되어 있다. High mobility group box 1 (HMGB1)은 원형위험신호물질 (prototypic alarmin)로서 임신 중 염증반응에 관여하여 조산발생에 중요한 역할을 한다고 알려져 있다. 이 연구는 융모양막염 및 자궁내염증이 있는 환자군과 정상군사이에 HMGB1의 발현 정도를 비교하고 microRNA-548이 HMGB1의 발현조절에 어떠한 역할을 하는지 알아보고자 시행되었다. 자궁내염증이 있는 환자 34명과 정상군 14명을 대상으로 자궁내 HMGB1의 발현 정도를 비교하였을 때 자궁내염증이 있는 환자군에서 유의하게 HMGB1의 발현이 높았다. 다음으로 자궁강을 둘러싸고 있는 태아막인 양막에서 HMGB1의 발현 정도를 비교하였을 때 융모양막염이 있는 환자의 양막에서 정상군과 비교하여 유의하게 HMGB1의 mRNA 및 단백질의 발현이 높게 나타났다. 또한 microRNA-548의 발현을 살펴보았을 때 융모양막염이 있는 환자군의 양막에서 정상군에 비하여 microRNA-548의 발현이 현저하게 낮았다. 다음으로 microRNA-548과 HMGB1의 상호관계를 규명하기 위하여 양막에서 양막상피세포를 분리 및 배양하여 실험을 진행하였다. 먼저 MicroRNA-548 inhibitor를 양막상피세포에 트랜스펙션하여 microRNA-548의 발현을 억제시켰을 때

양막상피세포에서 HMGB1의 발현이 유의하게 증가하였으며 양막상피세포에서 분비되는 HMGB1의 양도 증가하였다. 또한 양막상피세포를 배양하는 배지에 lipopolysaccharide를 처리하여 염증반응을 유발시켰을 때 MicroRNA-548 mimic을 트랜스펙션시킨 양막상피세포에서는 HMGB1의 발현 및 유리가 증가하지 않았다. 또한 microRNA-548 mimic을 트랜스펙션시킨 양막상피세포에서는 lipopolysaccharide로 염증반응을 유발시켰을 때 염증싸이토카인 및 케모카인 유리가 증가하지 않았다. 결론적으로 자궁내염증은 양막에서 microRNA-548을 억제시키고 이는 양막상피세포에서 HMGB1의 발현과 유리를 증가시킨다. 또한 microRNA-548은 양막상피세포에서 염증반응을 조절할 수 있다. 따라서 microRNA-548은 HMGB1의 발현을 조절함으로써 융모양막염이 있는 산모에서 조산을 유발하는데 역할을 한다.

핵심되는 말: 융모양막염, high mobility group box 1, 자궁내염증, microRNA-548, 조산