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Damage-Associated Molecular Pattern Markers HMGB1 and Cell-Free Fetal Telomere Fragments in Oxidative-Stressed Amnion Epithelial Cell-Derived Exosomes

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Abstract

Term labor in humans is associated with increased oxidative stress (OS) -induced senescence and damages to amnion epithelial cells (AECs). Senescent fetal cells release alarmin high-mobility group box 1 (HMGB1) and cell-free fetal telomere fragments (cffTF) which can be carried by exosomes to other uterine tissues to produce parturition-associated inflammatory changes. This study characterized AEC-derived exosomes under normal and OS conditions and their packaging of HMGB1 and cffTF.

Primary AECs were treated with either standard media or oxidative stress-induced media (exposure to cigarette smoke extract for 48 hours). Senescence was determined, and exosomes were isolated and characterized. To colocalize HMGB1 and cffTF in amnion exosomes, immunofluorescent staining and in situ hybridization were performed, followed by confocal microscopy. Next generation sequencing (NGS) determined exosomal cffTF and other cell-free amnion cell DNA specificity.

Regardless of condition, primary AECs produce exosomes with a classic size, shape, and markers. OS and senescence caused the translocation of HMGB1 and cffTF from AECs' nuclei to cytoplasm compared to untreated cells, which was inhibited by antioxidant N-acetyl cysteine (NAC). Linescans confirmed colocalization of HMGB1 and cffTF in exosomes were higher in the cytoplasm after CSE treatment compared to untreated AECs. NGS determined that besides cffTF, AEC exosomes also carry genomic and mitochondrial DNA, regardless of growth conditions.

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Sterile inflammatory markers HMGB1 and cffTF from senescent fetal cells are packaged inside exosomes. We postulate that this exosomal cargo can act as a fetal signal at term and can cause labor-associated changes in neighboring tissues.

Keywords

Fetal signals; parturition; microvesicles; DAMPs; inflammation; amniochorion

1. INTRODUCTION

Paracrine signals from the fetus that are indicative of fetal readiness prior to delivery are not well understood. Knowledge gaps exist in this area in our understanding of how signals from the fetus may reach the mother to affect the contractile state of the uterus and induce its transition from the relaxed to the laboring state to facilitate birth.

The functional mechanism of fetal-maternal communication in normal and preterm parturition is an inflammatory process. This inflammation is sterile or noninfectious in term parturition as well as in major subsets of preterm parturitions (Kusanovic et al., 2007; R. Romero et al., 2006; Roberto Romero et al., 2006; Romero et al., 2014, 2011, 2004). Investigation of the sources of sterile inflammation at term identified senescence-associated aging of fetal membranes as one of the causes. Fetal membranes undergo a telomere-dependent aging, which is correlated with in utero fetal growth. At term, senescence is accelerated in fetal membranes due to oxidative stress (OS) build up, which is an inevitable component of inflammation. Senescent fetal cells exhibit unique inflammatory biomarkers known as senescence-associated secretory phenotype (SASP) that are linked to initiation of labor (Behnia et al., 2016a; Menon et al., 2016a, 2016c; Poletini et al., 2015). Besides cytokines, chemokines, and other inflammatory mediators of SASP, sterile inflammation within the fetal membrane includes damage-associated molecular pattern (DAMPs) markers (Behnia et al., 2016b, 2015). DAMPs are molecules with defined intracellular functions, but outside of the cell, DAMPs are proinflammatory mediators. Examples of such DAMPs include high-mobility group box 1 (HMGB1), a nonhistone nuclear protein (Ciucci et al., 2011; Tang et al., 2011); HSP70, a chaperon protein (Andocs et al., 2015; Luong et al., 2012); fragments of cell-free DNA (Girard et al., 2014; Nadeau-Vallée et al., 2016; Phimister and Phillippe, 2014); telomere fragments (Poletini et al., 2015); and uric acid (Mulla et al., 2011). DAMPs are often recognized by pattern recognition receptors (PRRs) that are located in the cell membranes (Santoni et al., 2015). Ubiquitous expression enables PRRs to recognize and ligate DAMPs to promote signaling cascades causing inflammation, complement activation, cell necrosis, and apoptosis (Saïd-Sadier and Ojcius, 2012). We postulate that SASP and DAMPs constitute sterile inflammatory signals from the fetus with uterotonic properties and overwhelming production, and propagation of these signals between fetomaternal compartments constitute a novel paracrine signaling mechanism to initiate parturition.

Signals between fetomaternal compartments are likely propagated through either diffusion or via specific carriers. Exosomes, bioactive cell-derived vesicles (30–150 nm), function as

vectors to transport signals between tissues, including placenta (Record, 2014). Exosome cargo generally reflect the functional and physiologic state of the cell of origin (Stoorvogel et al., 2002). Recently, we have reported that fetal amnion cells release exosomes that can transport cellular metabolic byproducts, including DAMPs like HSP70 (Sheller et al., 2016). In our study, exosomes released from primary amnion epithelial cells (AECs—derived from normal term, not-in-labor fetal membranes) and AECs exposed to cigarette smoke extract (CSE; a well-characterized OS inducer that mimics OS in utero) showed distinct cargoes indicative of physiologic status of the cells. Bioinformatics analysis of exosome protein cargo showed inflammatory markers linked to NF- κ B signaling in normal AEC exosomes, whereas CSE-exposed cells had markers indicative of TGF- β pathway. The latter is indicative of epithelial-mesenchymal transition (EMT), which is also an inflammatory process under stress conditions (Sheller et al., 2016). We determined that distinct forms of inflammatory mediators can be transported by AEC-derived exosomes. Using animal models, we have shown that exosomes can traffic from the fetal to the maternal side either through diffusion or through the systemic route. Therefore, senescent fetal cell-derived exosomes can carry inflammatory signals and propagate them to the maternal side to cause functional changes, transitioning a quiescent uterus to its active contractile status.

Two of the DAMPs, HMGB1 and cell-free fetal telomere fragments (cffTF), have been determined as products of fetal cell senescence under OS. In vitro, in a feedback loop, both HMGB and cffTF increase fetal membrane senescence and SASP (Bredeson et al., 2014; Menon et al., 2016a; Poletti et al., 2015). Recently, recombinant HMGB1 has been shown to induce preterm labor in animal models (Gomez-Lopez et al., 2016), and both cell-free fetal DNA (cffDNA) (Phillippe, 2015) and cffTF are associated with preterm parturition in humans and animal models (Poletti et al., 2015). Therefore, SASP and DAMPs constitute sterile inflammation, and their functional synergy can promote overall uterine inflammatory load and mechanistic responsiveness, leading to labor. Although the 2 DAMPs mentioned here have functional roles in innate immune mechanisms and parturition, their localization in exosomes and any changes in their quantity in response to the physiologic status change of a cell have not been reported. Our objective was to localize HMGB1 and cffDNA, specifically telomere fragments, in AEC-derived exosomes and determine any changes in their concentrations in response to OS induced in vitro.

2. MATERIALS AND METHODS

Placentas for this study were obtained from John Sealy Hospital at The University of Texas Medical Branch (UTMB) at Galveston, TX, USA. No subjects were recruited or consented for this study as we used discarded placenta from normal term, not-in-labor cesarean sections as described previously (Sheller et al., 2016).

2.1. Isolation and culture of human AECs

The amniotic membranes obtained from term, not-in-labor cesareans were processed, as described previously (Lim et al., 2013; Menon et al., 2013; Sheller et al., 2016), within 15 minutes of delivery. All reagents and media were warmed to 37°C prior to use. AECs (n = 5) were cultured in T75 flasks containing complete media consisting of Dulbecco's Modified

Eagle Medium: Nutrient Mixture F-12 media (DMEM/F12; Mediatech Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), 10% Penicillin/Streptomycin (Mediatech Inc.), and 100- μ g/mL epidermal growth factor (EGF; Sigma-Aldrich) at 37°C, 5% CO₂, and 95% air humidity to 80% confluence. Cytokeratin-18 staining was used to confirm AEC specificity.

2.2. Stimulation of AECs with CSE

To induce oxidative stress in AECs, CSE was used as detailed in our prior studies (Menon et al., 2013; J Poletti et al., 2014; Jossimara Poletti et al., 2014) with modifications. Smoke from a single lit commercial cigarette (unfiltered CamelTM, R.J. Reynolds Tobacco Co, Winston Salem, NC) was bubbled through 25 mL of exosome-free media, consisting of DMEM/F12 supplemented with 5% exosome-free FBS. FBS (Sigma-Aldrich) was depleted of exosomes by ultracentrifugation at 100,000 g for 18 hours then filter-sterilized with 0.22- μ m filter (Millipore, MA, USA) (Kobayashi et al., 2014; Soo et al., 2012). The stock CSE was sterilized using 0.22- μ m Steriflip® filter unit (Millipore). CSE concentrate was diluted 1:50 in exosome-free media prior to use.

Cells were serum starved for 1 hour in DMEM/F12 with 5% pen/strep prior to treatment with exosome-depleted media (control), CSE containing media, or CSE containing media with the antioxidant N-acetyl cysteine (NAC; 15mM) and incubated at 37°C, 5% CO₂, and 95% air humidity for 48 hours. Untreated cells were grown under normal cell culture conditions and referred to as controls in the rest of this manuscript.

2.3. Senescence-associated β -galactosidase (SA- β -Gal) activity

Senescence was assessed with the commonly used biomarker senescence-associated β -galactosidase (SA- β -Gal) activity, adapted for flow cytometry (Cho and Hwang, 2011; Noppe et al., 2009) with modifications. Cells were incubated for 1 hour in complete DMEM growth medium supplemented with 100nM bafilomycin A1 (baf A1). After 1 hour, 5-dodecanoylamino fluorescein di- β -D-galactopyranoside (C₁₂FDG) was added for a final concentration of 6 μ M and incubated at 37°C, 5% CO₂, and 95% air humidity for 1 hour. Cells were harvested by trypsinization and centrifugation at 3000 g for 10 minutes at 4°C. The cell pellet was resuspended in 500- μ L Coulter DNA Prep Stain (Beckman Coulter, Indianapolis, IN) and run immediately on the CytoFlex flow cytometer (Beckman Coulter). Unstained, control AECs were used as negative controls for gating. Data analysis was performed using Cytexpert (Beckman Coulter).

2.4. Isolation of exosomes from control and CSE-treated amnion cells

After 48 hours, media from control and CSE-treated cells were collected and stored at -80°C until isolation. Media were thawed at 4°C overnight prior to isolation. Exosomes were isolated as described (Sheller-Miller et al., 2016; Sheller et al., 2016) with the following modifications. After centrifugation at 2000 g, the supernatant was transferred to Amicon Ultra 15 centrifugation filters (Millipore, Billerica, MA) and centrifuged at 2000 g for 90 minutes. The concentrate was collected and centrifuged at 10,000 g for 30 minutes. The supernatant was transferred to ultracentrifugation tubes and centrifuged at 100,000 g for 2 hours. The supernatant was discarded, and the pellet was resuspended in cold 1x PBS then

centrifuged at 100,000 g for 1 hour. The final pellet was resuspended in 1x PBS and stored at -80°C until use.

2.5. Exosome characterization

Exosomes were characterized for their size, quantity, shape, and exosome-specific markers prior to testing them for DAMPs.

2.6. Determination of exosome size and quantification

Exosome total size distribution and concentration was determined using the Nanosight NS300 (Malvern Instruments, Worcestershire, UK). Samples were diluted 1:500 in distilled water and run following the manufacturer's instructions. For all samples, we used a camera level of 15–16 and automatic functions for all postacquisition settings except for the detection threshold, which was fixed at 5. The camera focus was adjusted to make the particles appear as sharp dots. Using the script control function, three 30-second videos for each sample were recorded.

2.7. Determination of exosome shape using transmission electron microscopy (TEM)

Exosome shape was determined using a JEOL transmission electron microscope (TEM). The protocol for this experiment can be seen in our prior publications (Sheller-Miller et al., 2016; Sheller et al., 2016). Briefly, formvar/carbon-coated 300-mesh copper grids were treated with 10 seconds of hydrogen-oxygen plasma in a Gatan Solarus 950 plasma cleaning system (Gatan, Inc., Pleasanton, CA). Exosomes were dropped onto a grid and left to dry at room temperature for 10 minutes. After 3 washes in purified water, the exosome samples were negatively stained using phosphotungstic acid (PTA). The grids were dried at room temperature and viewed in a 120 keV JEM 1400 electron microscope (Jeol, Peabody, MA). A minimum of 15 frames were viewed per sample.

2.8. Western blot analysis

Exosomes in PBS were added 1:1 to 2x RIPA lysis buffer (50mM Tris pH 8.0, 150mM NaCl, 1% Triton X-100, and 1.0mM EDTA pH 8.0, 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktail and PMSF. Western blot samples then were processed as described previously (Sheller-Miller et al., 2016; Sheller et al., 2016). The following anti-human antibodies were used for Western blot: exosome marker CD9 (Abcam, Cambridge, United Kingdom) diluted 1:400 and Alix (Santa Cruz Biotechnology, Dallas, TX) diluted 1:500. We previously reported stem cell transcription marker Nanog as a consistent marker in AEC-derived exosomes, so we used Nanog (Cell Signaling, Beverly, MA) diluted 1:400 to confirm AEC specificity of exosomes.

2.9. Exosomal localization of HMGB1 using immunofluorescent (IF) staining

OS induction of AECs releases HMGB1 from the cells' nuclei to cytoplasm, where it is expected to be picked up by exosomes. To colocalize HMGB1 inside exosomes, IF staining was performed for both HMGB1 and exosome marker CD9. Once cells reached confluence, culture media were removed, and cells were rinsed with PBS, harvested with trypsin EDTA (Corning, Corning, NY), and centrifuged for 10 minutes at 3000 RPM. Cells were

resuspended in complete media and seeded on glass coverslips at a density of 30,000 cells per slip and incubated overnight. Cells, untreated and CSE-treated (48 hours), were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.5% Triton X, and blocked with 5% bovine serum albumin (BSA, Fisher Scientific, Waltham, MA) in PBS prior to incubation with primary antibodies HMGB1 (Cell Signaling) and CD9 (Abcam, Cambridge, United Kingdom) diluted 1:300 in 5% BSA overnight at 4°C. After washing with PBS, slides were incubated with Alexa Fluor 488- or 594-conjugated secondary antibodies (Life Technologies, Carlsbad, CA) diluted 1:1000 in 5% BSA for 30 minutes in the dark. Slides were washed with PBS, treated with NucBlue® Live ReadyProbes® Reagent (Life Technologies) for 2 minutes, and mounted using Mowiol 4–88 mounting medium (Sigma-Aldrich). Slides were dried at room temperature and stored in the dark until imaging.

2.10. Exosomal localization of cffTF using fluorescent in situ hybridization (FISH)

To colocalize cffTF in exosomes, cells were harvested as described above and seeded in Millicell EZ slides 4-well glass slides (Millipore, Billerica, MA) at a density of 65,000 cells/well overnight. After 48-hour treatment, cells were fixed and permeabilized with ice cold 100% methanol (stored at -20°C) for 10 minutes at -20°C and blocked with 5% BSA in PBS. Cells were incubated with primary antibody CD9 diluted 1:300 in 5% BSA at 4°C overnight while gently rocking. After washing with PBS, slides were incubated with Alexa Fluor 594-conjugated secondary antibody (Life Technologies) diluted 1:1000 in 5% BSA in the dark for 30 minutes. Cells were washed with PBS then refixed using 4% PFA and re-permeabilized with 70% EtOH overnight at 4°C. Wells of the slides were removed, and cells were dehydrated by 2-minute incubation in 85% and 100% ethanol. Slides were air-dried at room temperature for 10 minutes.

Leading strand (C-rich) telomere peptide nucleic acid (PNA) probe conjugated with Alexa Fluor 488 (PNA Bio, Newbury Park, CA) lyophilized powder was resuspended in 100-μL formamide to make a 50 μM stock, which was aliquoted and stored at -80°C. The stock was diluted in prewarmed hybridization buffer (Thermo Fischer) to a final concentration of 200 nM, and 50 μL was added to each prewarmed slide, covered with a coverslip, and heated at 85°C for 10 minutes. For in situ hybridization, slides were incubated at room temperature for 2 hours in a humidity chamber. After hybridization, slides were placed in 2x SSC with 0.1% Tween to remove the coverslips and washed twice in 2x SSC with 0.1% Tween heated to 55°C–60°C and again at room temperature. Slides were treated with NucBlue® Live ReadyProbes® Reagent (Life Technologies) for 2 minutes and then washed in 2x SSC, 1x SSC, and DI H₂O for 2 minutes each. Slides were dried at room temperature for 10 minutes then mounted using Mowiol 4–88 mounting medium (Sigma-Aldrich). Slides were dried at room temperature and stored in the dark until imaging.

2.11. Image processing and analysis

Confocal images were acquired using a Zeiss LSM-510 Meta confocal microscope with a 63 × 1.20 numerical aperture water immersion objective. The images were obtained using 3 excitation lines (364, 488, and 543), line emissions were collected with 385–470-nm, 505–530-nm, and 560–615-nm filters, respectively. All images were collected using 8-frame-Kallman-averaging with a pixel time of 2.51 μs, a pixel size of 160 nm, and an optical slice

of 1 μm . Z-stack acquisition was carried out with 0.8- μm z-steps. Image processing and analysis were performed with Metamorph 7.2 to set the low and high thresholds for measurement of immunofluorescent intensity. After discounting background in each channel we traced a linescan over the areas where we saw the highest intensity of colocalized signal. A graphic of raw fluorescence intensity vs calibrated distance in micrometers along the linescan (indicated by arrows on every figure) was plotted.

2.12. Exosomal localization of cffDNA

In addition to localizing HMGB1 and cffTF, we also determined the presence of cffDNA in exosomes derived from AECs grown under normal and OS conditions. The exosome pellets from control and CSE-treated cells were thawed on ice prior to DNA extraction. DNA was extracted following the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) for cultured cells protocol with the following modification. The DNA elution from exosomes was performed twice by adding 20- μL Buffer AE (TrisCl and EDTA) to the spin column, incubating for 1 minute, and then centrifuging for 1 minute at 7000 g. All DNA samples were quantified using a Qubit fluorimetric assay (Thermo Fisher Scientific, Waltham, MA) specific for double-stranded DNA.

2.13. NGS to determine exosomal cffTF and other cell-free amnion cell DNA specificity

2.13.1. Creation of NGS libraries for DNA-Seq—DNA libraries were created using the Nextera tagmentation technology (Illumina, San Diego, CA). Tagmentation uses a transposon-based approach that fragments the target DNA and introduces a partial adapter sequence in a single step. All DNA samples were quantified using a Qubit fluorometric assay (Thermo Fisher Scientific). DNA quality was assessed using a high-sensitivity DNA chip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Creation of DNA libraries was performed using 50 ng of genomic DNA and Nextera tagmentation reagents as recommended by the manufacturer. Limited PCR (5 cycles: $95^{\circ}\text{C} \times 10$ seconds/ $62^{\circ}\text{C} \times 30$ seconds/ $72^{\circ}\text{C} \times 3$ minutes) amplification was performed to complete the adapter sequence and index the final library. All NGS libraries were indexed independent of the planned complexity of the sequence analysis. The final concentration of all NGS libraries was determined using a Qubit fluorometric assay, and the DNA fragment size of each library was assessed using a DNA 1000 high-sensitivity chip and an Agilent 2100 Bioanalyzer.

2.13.2. NGS data analysis—We created an artificial telomere sequence by concatenating 50 copies of the consensus sequence TTAGGG. Adapter sequences were trimmed from the reads in fastq format using the program Trimmomatic, version 0.36, without quality trimming. Reads were then aligned in paired end format to the telomere reference using Bowtie2, version 2.2.5, in the local alignment mode with default parameters. The number and percentage of mapped reads were reported in the Bowtie2 output (Bolger et al., 2014; de Menezes-Neto et al., 2015). Circular representations of the readings of fragments along each chromosome in the whole-genome and mitochondria were created using Circos (Krzywinski et al., 2009).

2.14. Statistical analysis

SPSS software (IBM, Armonk, NY) was used for statistical evaluation. Samples were analyzed using independent sample *t* test, and a *P* value less than .05 was considered statistically significant.

3. RESULTS

3.1. CSE induces cellular senescence in primary AECs

AEC specificity was confirmed using cytokeratin-18 staining. To determine cellular senescence, a determining factor in HMGB1 and cffTF release, control and CSE-treated cells were analyzed for SA- β -Gal activity using flow cytometry (Cahu and Sola, 2013; Cho and Hwang, 2011; Debacq-Chainiaux et al., 2009; Noppe et al., 2009). As shown in Figure 1, control cells (9.5%) had significantly ($P < .0001$) less senescent cells compared to CSE-treated cells (38.0%). This finding confirmed our prior histology-based reports that CSE causes AEC senescence.

3.2. Characterization of exosomes from control and CSE-treated AECs

Prior to localization of HMGB1 and cffTF in exosomes, we determined the characteristics of exosomes derived from control and CSE-treated AECs. TEM studies (Figure 2A) showed, regardless of treatment, amnion exosomes exhibited cup-shaped morphology and a size distribution of 50–150 nm (Sarker et al., 2014; Winther and Thermofischer, 2015). Nanoparticle tracking analysis was performed to confirm size distribution and quantify the number of exosomes per sample (Figure 2B). There was no significant difference ($P = .474$) seen in the size distribution of exosomes derived from control (138.6 nm) and CSE-treated (137.8 nm) AECs. Additionally, after quantifying the number of exosomes in each prep, we calculated the number exosomes released per cell in each experiment. We did not see significant difference ($P = .53$) in number of exosomes secreted in control (2727 exosomes per cell) and CSE-treated (2926 exosomes per cell) cells.

A Western blot was performed to characterize common exosome markers and cell-type-specific markers in each sample (Figure 2C). Regardless of condition, AEC-derived exosomes were positive for exosome markers CD9 and Alix, as well as embryonic stem cell marker Nanog.

3.3. OS causes exosome localization of HMGB1

HMGB1, a nonhistone nuclear protein, was localized in the nucleus (green staining) in control cells (Figure 3A). OS induced by CSE, however, caused translocation of HMGB1 from the nucleus to cytoplasm (Figure 3B). This translocation was inhibited by the antioxidant N-acetyl cysteine (NAC) (Figure 3C), suggesting OS-induced nuclear injury and HMGB1 release. Red staining in the cells represent CD9⁺ exosomes. Next, we determined colocalization of HMGB1 within the exosomes in both control and CSE-treated AECs. Control amnion cells displayed only a few overlapping spots in a cell, whereas CSE treatment produced cytoplasmic localization of HMGB1 and multiple areas of colocalization (Figure 4A and 4B). A linescan confirmed that colocalization of HMGB1 in exosomes was higher in the cytoplasm after CSE treatment compared to controls AECs. This finding

confirms that exosomes and HMGB1 are colocalized, and they can potentially escort off the cell together.

3.4. OS causes exosome localization of cffTF

FISH was performed to colocalize cffTF and exosome marker CD9. Under standard cell culture conditions, telomeres are localized in the AEC nucleus (Figure 5A). Treatment of AECs with CSE caused fragmentation of telomeres (as reported previously) and telomere fragment translocation from the nucleus to the cytoplasm (Figure 5B), which was inhibited by treatment with the antioxidant NAC (Figure 5C). A linescan was performed to determine colocalization of telomere fragments with exosome marker CD9 (Figure 5D and 5E). As shown in Figure 5E, colocalization of cffTF in exosomes was higher in CSE-treated AECs compared to control.

3.5. Exosomes released from AECs contain genomic and nongenomic DNA

DNA extracted from control and CSE exosomes was analyzed by high throughput sequencing. DNA sequencing was done to confirm the above reported findings using FISH. By creating an artificial telomere sequence using 50 copies of the consensus sequence TTAGGG, we determined that CSE exosomes carry nongenomic DNA and their telomere fragment specificity. Quantitative assessment of any differences in cffTF between normal and CSE-derived AEC exosomes were not attempted in this approach.

In addition to determining cffTF in exosomes, we also report the presence of genomic DNA in exosomes. Reads from control and CSE AEC exosomes were aligned to the whole genome (Figure 7A). While we did not find an overrepresentation of a specific chromosome in either treatment, we did see that CSE exosomes had significantly higher GC content than control exosomes ($P = .023$). Control and CSE DNA reads were also aligned to mitochondrial DNA (Figure 7B), and we report the packaging of mitochondrial DNA in exosomes derived from AECs. Previously, we reported that DNase digestion is not necessary in AEC exosomes studies because DNA fragments sticking to exosomes are not seen in our preparations (Sheller et al., 2016).

4. DISCUSSION

Fetal tissue-derived paracrine signals are rarely investigated during parturition. Based on our ongoing investigation, we have reported telomere-dependent fetal membrane senescence as a factor associated with human and murine parturition, resulting in aging of fetal membranes (Behnia et al., 2016a, 2015; Bonney et al., 2016; Bredeson et al., 2014; Menon et al., 2016c; Poletini et al., 2015). Aging of the fetal membranes correlates with fetal growth and, therefore, can be considered one of the signals from the fetus to the mother to initiate labor and delivery. Senescence is an inflammatory condition, and we have previously reported that signals arising from senescent fetal tissues are proinflammatory (Behnia et al., 2016a, 2016b, 2015; Menon, 2014; Menon et al., 2016b, 2013). These signals include SASP and DAMPS, both of which can traverse through various feto-maternal compartments and potentially cause inflammatory overload, a functional feature associated with parturition. Trafficking of these signals is likely carried out by microvesicles, such as exosomes, and our

prior reports have shown that exosomes are produced from senescent fetal cells and carry inflammatory signals (Sheller et al., 2016). These exosomes can either diffuse through tissue layers or reach their destination via the systemic route. Animal model experiments have confirmed trafficking of fetal exosomes through both mechanisms in which intraamniotic injection of exosomes resulted in their localization in both maternal blood as well as uterine tissues (Sheller-Miller et al., 2016). Thus, senescent fetal cells can package inflammatory mediators inside exosomes and transport them to other uterine destinations.

So far, our studies have been restricted to characterizing senescence, senescence-associated inflammation, and production of exosomes under normal and senescent conditions. We have not yet determined any specific cargo contents in AEC-derived exosomes, specifically the DAMPs HMGB1 and cffTF, both of which are proinflammatory. This study localized HMGB1 and cffTF inside fetal cell-derived exosomes. OS induced by CSE caused nuclear injury, an expected event, resulting in the release of HMGB1 and cffTF to the cytoplasm. In the cytoplasm, these molecules were packaged inside AEC-derived exosomes. Our data did not show any increase in the number of exosomes between control and OS groups. However, specific cargo contents were differentially packaged inside exosomes after OS induction. Both HMGB1 and cffTF were colocalized inside the exosomes in OS-exposed AEC-derived exosomes, and minimal colocalization was seen in control cell-derived exosomes. The OS effect was inhibited after NAC treatment, confirming the impact of OS on exosome cargo packaging. These data further confirm the notion that exosome cargo, not their quantity, reflects the physiologic state of the cell of its origin. In our study, OS caused increased packaging of 2 DAMPs inside exosomes. Although there are several DAMPs reported to be released from fetal cells, our study was restricted to HMGB1 and cffTF due to their functional roles in enhancing inflammation.

Both HMGB1 and cffTF activate stress signaler p38 MAPK activation (Bredeson et al., 2014; Poletini et al., 2015). HMGB1 and cffTF, in a feed-forward loop, enhance fetal cell senescence and tissue injury and augment HMGB1 and cffTF production and packaging in fetal exosomes. Release of these exosomes results in an increased inflammatory load in the fetal compartments and likely at distant sites. Inflammation resulting from DAMP-induced senescence was inhibited by SB203580, a p38 MAPK inhibitor, confirming the activation of senescence pathway (Bredeson et al., 2014; Poletini et al., 2015). Multiple reports have shown the contribution of HMGB1 in parturition at term and preterm (Behnia et al., 2016a, 2015; Gomez-Lopez et al., 2016; Phillippe, 2015; Romero et al., 2011), and other laboratories have confirmed its release due to fetal membrane injury (Nadeau-Vallée et al., 2016; Romero et al., 2011). Similarly, we have also reported the role of cffTF in causing OS, senescence, and inflammation in pregnant animal models (Poletini et al., 2015). In an ongoing investigation, we have seen that HMGB1 and cffTF cause COX-2 and connexin-43 expressions in myometrial cells, suggesting that if these DAMPs reach quiescent myometrium, they may lead to parturition related changes.

In addition to cffTF, we also report cffDNA as exosome cargo. cffDNA carried by fetal cell exosomes may also cause parturition-associated inflammatory functions as reported by Phillippe et al. It is shown to exert its proinflammatory functions through TLR-9. Isolation of cffDNA from maternal blood and its association with preterm parturition have also been

reported (Menon et al., 2012; Nadeau-Vallée et al., 2016; Phillippe, 2015; Phimister and Phillippe, 2014). The packaging of cfDNA in exosomes may ensure better delivery and functional activity since it is protected inside the exosomes. Although the specific nature of genomic DNA carried by exosomes is unclear in our study, our data demonstrate a novel mechanism of transportation of cfDNA.

Data from this study support that exosomes can be carriers of these 2 DAMPs where they may exert a proinflammatory function as mentioned above. This OS-induced senescence model and exosome cargo of inflammatory mediators is one of the mechanisms by which the fetus may communicate with the mother. CSE was used in our study to cause OS, and, although it mimics OS at term parturition, the exact nature of OS experienced by fetal tissues at term may differ. OS-induced pregnancy-associated risk factors can cause premature senescence of fetal membranes, which may result in the release of exosomes carrying SASP and DAMPs that can reach maternal tissues to signal initiation of preterm parturition. OS-associated and telomere-dependent senescence has been reported in preterm premature rupture of the membranes (Menon, 2014; Menon and Fortunato, 2004), a major subset of spontaneous PTB. Initiation of labor in this subset likely results from exosomal cargo that carry OS-induced senescent cell-derived signals.

We did not investigate the functional role of exosomes in uterine tissues although that is an ongoing investigation in our laboratory. In addition to the 2 DAMPs reported here, other DAMPs and still unreported fetal-derived mediators may perform similar functions. Characterization of exosome cargo using OMICs approaches is expected to reveal more fetal signals. Our study was restricted to amnion-derived signals. We have shown that chorion also undergo senescence and can produce SASP and DAMPs. However, the chorion-derived signaling is also not investigated in this report.

We provide some additional information to the ongoing postulates of fetal tissue injury resulting from senescence at term and its potential contribution to parturition signals. DAMPs released from senescent fetal membranes in response to OS are packaged inside exosomes compared to normal cells and can be delivered. Inflammatory properties of these DAMPs suggest that exosomal delivery to maternal uterine tissue may prompt parturition-associated changes. Functional characterization of fetal exosome-derived signals is expected to provide more knowledge on fetal signaling during parturition.

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HIGHLIGHTS

- Oxidative stress induces primary amnion cells to undergo cellular senescence.
- Senescence results in damage-associated molecular pattern (DAMPs) release.
- Senescence causes nuclear to cytosolic migration of DAMPs HMGB1/telomere fragments
- Cytoplasmic HMGB1 and telomere fragments are packaged inside exosomes.
- HMGB1 and telomere fragments represent sterile inflammation propagated via exosomes.

Figure 1. Flow cytometry of SA-β-Gal activity on amnion cells cultured under standard (control) and OS (CSE) conditions

(A) Representative flow cytometry histograms of SA-β-Gal activity on amnion cells cultured under standard (control) and OS (CSE) conditions. (B) Bar graphs show flow cytometry analysis of SA-β-Gal activity for amnion cells cultured under standard (control) and OS (CSE) conditions (n = 6). *P < .0001

Figure 2. Characterization of exosomes released from amnion cells cultured under standard (control) and OS (CSE) conditions

(A) Electron microscopy shows cup/round-shaped exosomes regardless of treatment. (B) Western blot analysis for CD9, Alix, (exosome markers) and Nanog (amnion stem cell marker). (C) Representative images from nanoparticle tracking analysis (NTA) of control and CSE exosomes. All our preparations showed particles < 140 nm.

Figure 3. Localization of HMGB1: Confocal whole cell images of cells double immune-labelled for CD9 (red) and HMGB1 (green) markers

(A) Untreated amnion cells with nuclear HMGB1. (B) CSE-treated amnion cells with cytoplasmic HMGB1. (C) Cotreatment of amnion cells with CSE and the antioxidant N-acetyl cysteine (NAC) inhibit translocation of HMGB1.

Figure 4. Colocalization of HMGB1 in exosomes

(A) Colocalization of HMGB1 and CD9+ exosomes in untreated amnion cells compared to CSE treated amnion cells. (B) Confocal xy-planes of the z-stack. Intensity profile graphs show the topographical profile of the pixel intensity levels of each antibody labelling along the freely positioned arrow. The maximum height represents the brightest possible pixel in the source image.

Figure 5. Localization of cffTF: Confocal whole cell images of cells double immune-labelled for CD9 (red) and telomere fragments (green)
(A) Untreated amnion cells with telomere fragments in the nucleus. (B) CSE-treated amnion cells with telomere fragments in the cytoplasm. (C) Cotreatment of amnion cells with CSE and the antioxidant NAC have nuclear localization of telomere fragments.

Figure 6. Colocalization of cffTF in exosomes

(A) Colocalization of telomere fragments in untreated amnion cells compared to CSE treated amnion cells. (B) Confocal xy-planes of the z-stack. Intensity profile graphs show the topographical profile of the pixel intensity levels of each antibody labelling along the freely positioned arrow.

Figure 7. Circular view of the readings of fragments along each chromosome in the (A) whole-genome and (B) mitochondria sequencing analysis of exosomal DNA isolated from amnion cells grown under standard (control) and OS (CSE) conditions.