OBSTETRICS

Cyclic-recombinase-reporter mouse model to determine exosome communication and function during pregnancy

Samantha Sheller-Miller, PhD; Kyungsun Choi, PhD; Chulhee Choi, MD, PhD; Ramkumar Menon, MS, PhD

BACKGROUND: During pregnancy, feto-maternal communication can be mediated through extracellular vesicles, specifically exosomes, 30- to 150-nm particles released from each cell. Exosomes carry cellular signals, and traffic between fetal and maternal tissues to produce functional changes in recipient cells. Exosomes may function as a biomarker indicative of the physiologic status of their tissue of origin. These properties of exosomes during pregnancy are not well studied.

OBJECTIVE: To test exosome trafficking and function, we used a transgenic mouse model containing membrane-targeted, red fluorescent protein tdTomato and enhanced green fluorescent protein cyclic recombinase–reporter construct expressed only in fetal tissues. This model allows fetal tissues and their exosomes to express tdTomato under normal conditions or green fluorescent protein if fetal tissues are exposed to cyclic recombinase that will excise tdTomato. As maternal tissue remains negative for this construct, tdTomato/green fluorescent protein expression and their switching can be used to determine fetal-specific cell and exosome trafficking.

MATERIALS AND METHODS: tdTomato/green fluorescent protein–homozygous male mice were mated with wild-type females to have all fetal tissues express the tdTomato/green fluorescent protein allele. Red fluorescence due to tdTomato expression of the tdTomato/green fluorescent protein allele in fetal tissues (placenta, fetal membranes) was confirmed by confocal microscopy on embryonic day 16. Localization of fetal exosomes in maternal uterine tissues were performed by immunostaining for exosome marker CD81 and tdTomato expression followed by confocal microscopy. Fetal exosomes (tdTomato–positive) in maternal plasma were immunoprecipitated using anti–red fluorescent protein tdTomato, followed by confirmation with flow cytometry. To further illustrate the fidelity of fetal exosomes in maternal samples, exosomes bioengineered to contain cyclic recombinase (1.0×10^{10} exosomes) were injected intraperitoneally on embryonic day 13. On embryonic day 16, fetal (placenta and fetal membranes) tissues were imaged to show tdTomato–to–green fluorescent protein transition. The green fluorescent protein–expressing exomes were localized in maternal tissues (confocal microscopy) and plasma (flow cytometry).

RESULTS: Mating between a male with the tdTomato/green fluorescent protein construct and a null female resulted in fetal tissues and their exosomes expressing tdTomato positivity. Total fetal exosomes in maternal plasma was about 35%. tdTomato–positive exosomes were isolated from maternal plasma and immunostaining localized tdTomato–positive exosomes in maternal uterine tissues. Maternal intraperitoneal injection of cyclic recombinase–enriched exosomes crossed placenta, excised tdTomato from the tdTomato/green fluorescent protein construct in the fetal tissues, and caused green fluorescent protein expression in fetal cells. Furthermore, green fluorescent protein–positive exosomes released from fetal cells were isolated from maternal blood.

CONCLUSION: In this pilot study, we report feto-maternal and maternal–fetal trafficking of exosomes indicative of paracrine signaling during pregnancy. Exosomes from the maternal side can produce functional changes in fetal tissues. Trafficking of exosomes suggests their potential role in pregnancy as biomarkers of fetal functions and usefulness as a carrier of drugs and other cargo to the fetal side during pregnancy. Isolation and characterization of fetal exosomes can advance fetal research without performing invasive procedures.

Key words: extracellular vesicles, microvesicles, placenta, pregnancy, signaling, TdTomato, uterus

Extracellular vesicles, specifically exosomes,[1] have recently received plenty of attention from the scientific community as intercellular communication signals and for their roles in both physiological and pathophysiological functions in cells.[2–5] Exosome size facilitates easy trafficking between local and distant sites. Their cargo can also regulate the phenotype of the target cell. Hence, exosomes are considered to be a central component of paracrine cell–cell communication.[6,7] Since discovery, exosomes have been widely investigated for functional roles in neurodegenerative diseases,[2,8,9] viral and bacterial pathogenicity,[10–12] and cancer progression.[13–15] Exosome cargo reflects the physiological state of their cells of origin, a property that has been exploited using exosomes as a biomarker for various pathologies.[15,16]

Exosomes as intercellular communicators have not been well reported in reproductive biology. Unlike other medical disciplines, pregnancy and parturition not only involve constant interindividual communication between the mother and her fetus, but also intercellular communication between intrauterine tissues. Both fetal and maternal endocrine and immune regulators are well-documented biochemical mediators of communication[17,18]; however, ambiguity exists in their role as the

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AJOG at a Glance

Why was this study conducted?

Paracrine signaling and communication via exosomes are not tested during pregnancy or parturition. This study was conducted to determine feto-maternal exosome trafficking and function during pregnancy.

Key findings

The following were observed in this study: exosomes can traffic between the fetus and the mother; maternal exosomes reaching the fetal tissues can produce functional changes; and fetal-specific exosomes can be isolated and characterized in maternal liquid biopsy specimens.

What does this add to what is known?

This study showed paracrine communication via exosome between feto-maternal tissues during pregnancy. In addition, fetal exosome trafficking to the maternal side is a useful indicator of fetal physiology that can be measured in minimally invasive maternal samples. Likewise, trafficking of exosomes from the maternal side to the fetus to cause functional impact is suggestive of potential usefulness of exosomes-based delivery of specific molecules (eg, drug) during pregnancy.

only communication signals during pregnancy. Advances in exosome biology have implied exosome mediation in feto-maternal communications. Desrochers et al recently reported the functional role of embryonic stem cell–derived exosomes in implantation and trophoblast development.[19] Expression of placental alkaline phosphatase (PLAP) on exosomes, specifically from human placenta, has helped their characterization and determination of their roles in immune protection, nutrient transport, and pregnancy maintenance.[20–22] Biomarker potential of PLAP-positive exosomes during various pregnancy complications has also been reported.[23–26]

Our prior reports on exosomes as paracrine signalers during parturition[27–29] have shown that fetal cell–derived exosomes injected intra-amniotically can traffic from the fetal compartment (amniotic fluid) to maternal tissues in murine models of pregnancy.[29] In addition, in murine models, maternal plasma exosomes from gestation day (E) 18 (normal gestational period, 19–21 days) that are rich in inflammatory mediators, caused preterm birth when injected intraperitoneally on E15. Preterm birth was not evident in animals injected with exosomes from E9, which did not contain inflammatory cargo. Animals that delivered preterm showed localized inflammation as well as increased expression of labor-associated markers in uterine tissues. However, there was a lack of systemic progesterone withdrawal noted, which is the proposed mechanism of labor in this animal model. This suggests a pro-labor paracrine functional role for exosomes in this model. The plasma exosomes used for this study contained both maternal and fetal exosomes. Therefore, contributions specifically by fetal or maternal exosomes in triggering parturition are still unclear.

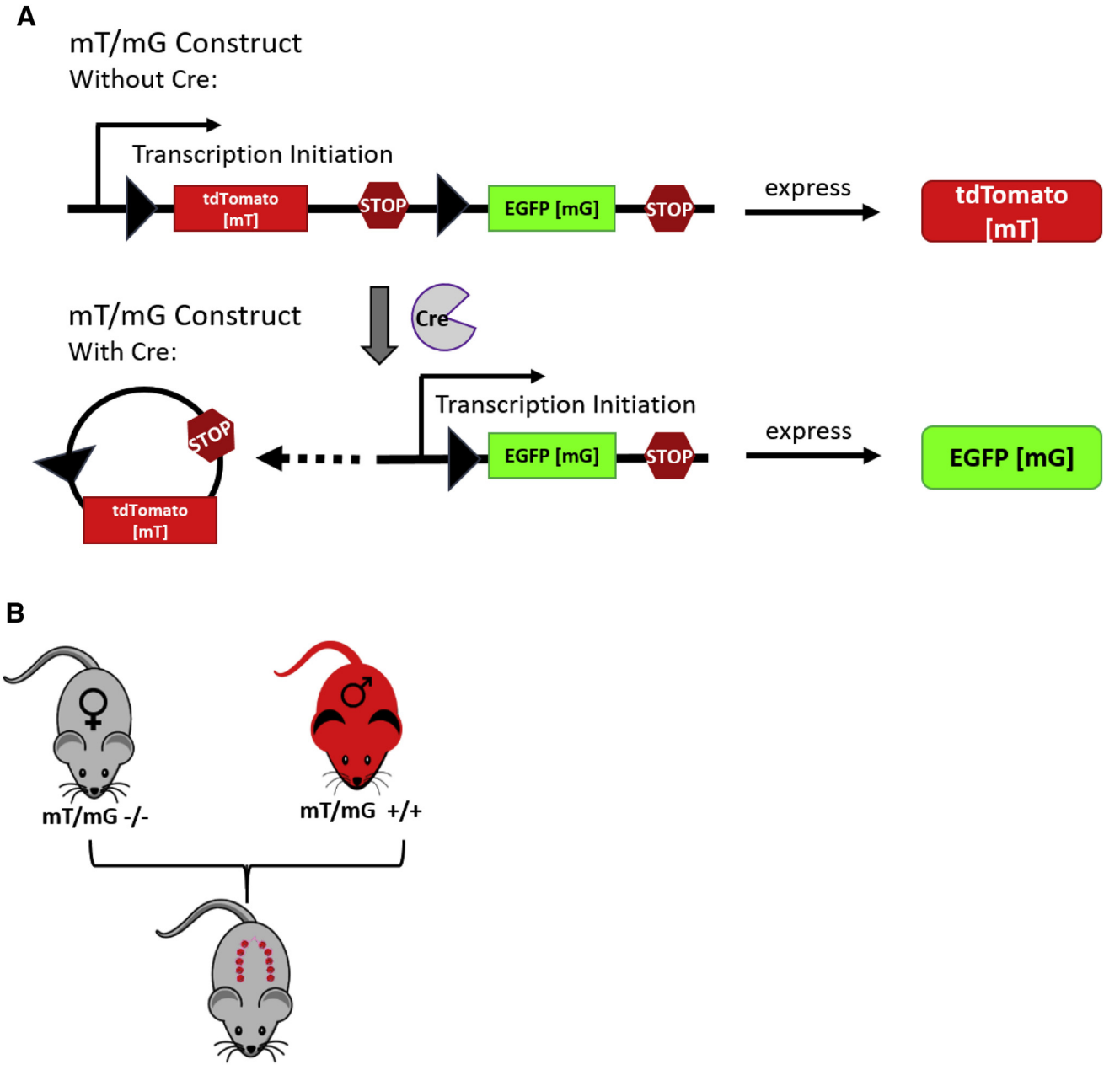
This study aimed to determine feto-maternal communication through exosomes and functional changes, if any, that can be produced by exosomes on either side. For this, we established a murine model in which fetal cells, and thus exosomes from these cells, express a specific marker that is absent in maternal tissues, allowing us to distinguish and to isolate fetal and maternal exosomes. A transgenic male mouse with a membrane-targeted, 2-color fluorescent cyclic recombinase (Cre)–reporter allele was used, where tandem dimer tomato (TdTomato [mT]) fluorescence is expressed in the plasma membrane of all cells and tissues. A system was developed in which, in the presence of Cre, mT is excised and cells express membrane-localized enhanced green fluorescent protein (EGFP [mG]) that has replaced mT (Figure 1A).[30] Mating a wild-type female with a male carrying this construct produced fetal tissues expressing this protein. Using this approach, we isolated and characterized fetal-specific exosomes from maternal blood. Using engineered exosomes containing Cre,[31] we determined that maternal injection of Cre-enriched exosomes can cross placental barriers, reach fetal tissues, and produce functional changes whereby mT expressing tissues were transitioned to express mG. Herein, we demonstrate feto-maternal trafficking of exosomes, isolation of fetal-specific exosomes from maternal plasma, and functional changes produced by exosomes crossing the placental barrier to fetal tissues.

Materials and Methods

Animal care

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Medical Branch, Galveston. Mice were housed in a temperature- and humidity-controlled facility with 12:12-hour light/dark cycles. Regular chow and drinking solution were provided ad libitum. We used a transgenic C57BL/6J mouse with a plasma membrane–targeted, 2-color fluorescent Cre-reporter allele (Figure 1A), in which tandem dimer Tomato (mT) fluorescence is expressed in the cell membrane of all cells and tissues, but, in the presence of Cre, mT is excised and cells express membrane-targeted EGFP (mG) on the cell membrane (stock 007676, Jackson Laboratory, Bar Harbor, ME). The mT/mG transgenic mouse model uses 2 highly photostable fluorescent proteins with low cytotoxicity. The red fluorescent protein mT, which is expressed in the absence of Cre, has increased brightness and photostability compared to other red fluorescent proteins, as well as deeper penetration using longer wavelengths of light,[30] allowing for easier in vivo imaging of intrauterine tissues. Breeding was performed in our

FIGURE 1
Cyclic recombinase (Cre)—reporter transgenic mouse model



A, To study feto-maternal exosome communication, we developed an animal model that uses a 2-color fluorescent Cre-reporter allele (mT/mG construct), which has a membrane-targeted tandem dimer Tomato (mT) red fluorescent protein (red) in all cells and tissues, whereas enhanced green fluorescent protein (mG) is not expressed (**top**). In the presence of Cre, mT is excised and cells express mG, which replaces mT expression (**bottom**). **B,** Female wild-type (WT) mice were mated with males homozygous for the mT/mG construct, so that all fetal tissues express the mT/mG construct and maternal tissues are negative.

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facility in which wild -type (WT) C57BL/6J (stock 000664, Jackson Laboratory) females, 8–12 weeks old, were mated with males homozygous for the mT/mG construct (Figure 1B). Female mice were checked daily between 8:00 and 9:00 am for the presence of a vaginal plug, indicating gestation day (E) 0.5. Females positive for a plug were housed separately from the males, their weight was monitored, and a gain of at least 1.75 g by E10.5 confirmed

pregnancy. Animals were sacrificed by CO_2 inhalation according to the IACUC and American Veterinary Medical Association guidelines prior to tissue collection.

Generation and isolation of Cre recombinase–loaded exosomes

HEK293T cells were transfected with Cre-mcherry-CRY2 and CIBN-EGFP-CD9 expression vectors. Stable cell lines were generated from single cells with red (mCherry) and green (GFP) fluorescence that were cultured until confluence. Cells were incubated with serum-free medium for 48 hours under 488-nm blue-light illumination.[31] The medium, as well as medium from HEK293T cells cultured under standard conditions (naive), was collected and filtered through a syringe filter (0.2 μm, Sartorius, Goettingen, Germany) to remove cellular debris. Exosome isolation and purification were performed by tangential flow filtration and size exclusion chromatography. Exosome particles were measured by nanoparticle tracking analysis (ZetaView, Particle Metrix, Germany).

Injection of Cre-enriched exosomes to determine maternal-to-fetal exosome trafficking

On E13, pregnant WT mice were intraperitoneally injected with 1 of the following: phosphate-buffered saline (PBS), naive exosomes, or Cre-enriched exosomes (1×10^{10} exosomes in 250 μL) (Figure 1B). Naive exosomes are exosomes produced by cells under standard conditions and were used as an exosome control. E13 was selected as the date for injection to maximize the red-to-green changes that occur in the fetal tissues. Because E13 is after placental development but before initiation of senescence of the mouse fetal membranes,[32] exosomes would need to cross the placental barrier, and fetal cells responding to Cre recombinase would still be proliferative and not yet undergoing senescent changes. At 72 hours postinjection (E16), females were sacrificed for plasma and tissue collection.

Immunofluorescent imaging for mT and mG signal in fetal and maternal tissues

Tissue samples were collected in 4% paraformaldehyde and stored overnight (4°C) before being washed twice with 1× PBS and transferred to a 15% sucrose solution overnight (4°C). Samples were then transferred to 30% sucrose and stored at 4°C until they were embedded in optimal cutting temperature compound and cut into 5-μm sections. Sections were incubated at room temperature for 30 minutes, then washed twice in water to remove optimal cutting temperature compound. Sections were incubated with DAPI for nuclear staining for 2 minutes at room temperature, then washed twice in water. To reduce the autofluorescence, tissues were incubated for 10 seconds with TrueVIEW Autofluorescence Quenching Kit (Vector Laboratories, Burlingame, CA), then washed twice with 1× Tris-buffered saline–Tween 20 (TBS-T). Slides were air dried at room temperature for 10 minutes and then mounted using Mowiol 4-88 mounting medium.

Immunofluorescent staining for mT colocalization in maternal tissues

For exosome marker CD63 colocalization with mT, after removing OCT, sections were incubated with blocking buffer (3% bovine serum albumin in Tris-buffered saline and 0.1% Tween 20) for 1 hour at room temperature in a humidity chamber. Blocking buffer was removed and Alexa Fluor 647 conjugated anti-CD63 (NBP234779Y, Novus Biologicals, Littleton, CO) diluted 1:250 in blocking buffer. After 1 hour of incubation at room temperature in a humidity chamber, slides were washed twice with Tris-buffered saline and 0.1% Tween 20, stained with DAPI, incubated with TrueVIEW, and mounted as described above.

Confocal images were captured using a Zeiss LSM 880 with Airyscan (Zeiss Corporation, Oberkochen, Germany). Brightness, contrast, and smoothing were applied to the entire image, and colocalization of mT-expressing exosomes and CD63 was performed using FIJI (open source).

Maternal plasma collection

Maternal blood was collected by cardiac puncture in tubes containing ethylenediamine tetraacetic acid (EDTA; Becton Dickinson, Franklin Lakes, NJ), and plasma was harvested after centrifugation (2000 *g* for 10 minutes at 4°C) and then stored at −80 °C.

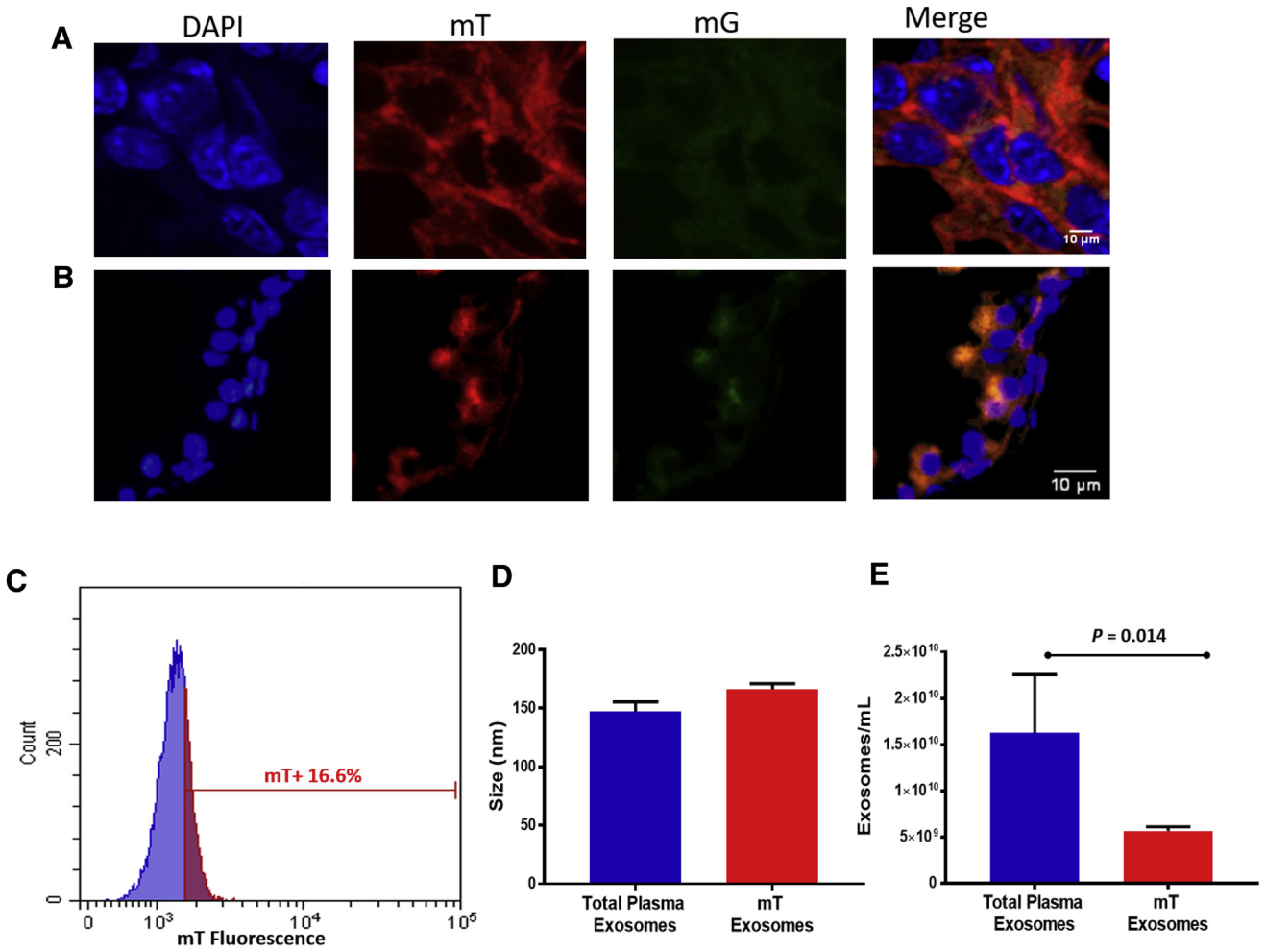
Maternal plasma exosome isolation

Exosomes from maternal plasma were isolated as described previously, with modifications.[33–35] Briefly, plasma samples were thawed on ice, diluted to 1.5 mL in cold 1× PBS, and centrifuged at 2000 *g* for 10 minutes at 4°C. Supernatants were transferred to clean microcentrifuge tubes and then filtered through Nalgene Syringe Prefilter Plus (Thermo Fisher, Waltham, MA). After filtration, samples were centrifuged at 10,000 *g* for 30 minutes. The supernatant was transferred to ultracentrifuge tubes and centrifuged at 100,000 *g* for an additional 2 hours. The supernatant was subsequently discarded, and the pellet was resuspended in 100 μL of cold 1× PBS. The final pellet was passed through an Exo-spin column (Cell Guidance Systems, St. Louis, MO) for size exclusion chromatography following manufacturer instructions. Samples were aliquoted and stored at −80°C.

Exosome immunoprecipitation and flow cytometry analysis

Immunoprecipitation (IP) of mT- or mG-expressing exosomes was performed using Exo-Flow (System Biosciences, Mountain View, CA) as described previously,[27] with modifications. Briefly, magnetic beads coated with streptavidin were incubated with 500 ng/mL either biotinylated anti-mT (ab3477, Abcam, Cambridge, UK) or biotinylated anti-mG (ab6658, Abcam) for 2 hours on ice, flicking intermittently to mix. Beads were washed and then incubated with 100 μL exosomes and rotated overnight at 4°C. The following day, before removing exosomes from the beads, we validated the IP using flow cytometry. Beads were washed, and then stained using the reversible Exo-FITC exosome stain according to

FIGURE 2
mT is expressed in mouse fetal tissues



A and **B,** Placenta (**A**) and fetal membranes (**B**) from pregnant wild-type (WT) mice were collected on E16 and imaged for membrane-targeted tdTomato (mT) expression. Scale bars, 10 μm. **C**, Maternal plasma exosomes were isolated and analyzed for mT+ exosomes using bead-coupled flow cytometry. **D** and **E**, Total maternal plasma exosomes and mT-expressing exosomes removed from the beads were analyzed using nanoparticle tracking analysis for size (**D**), which was not significantly different, and concentration (**E**), which was significantly decreased in mT+ exosomes compared to total plasma exosomes ($P = 0.014$). All data are from n = 4 and expressed as mean ± standard error of the mean. $P \leq 0.05$ was considered significant.

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manufacturer instructions. Negative controls incubated with antibody/Exo-FITC stain and without exosomes were used for gating, applied according to manufacturer instructions. Data analysis based on FITC signal shift of 10,000 events per sample was performed using CytExpert (Beckman Coulter). Once flow cytometry validated our IP, exosome elution buffer provided in the Exo-Flow kit was used to remove fluorescein isothiocyanate (FITC) stain and collect exosomes from beads following the manufacturer's instructions. The supernatant containing exosomes was collected for nanoparticle tracking analysis (mT exosomes only) and dot blot.

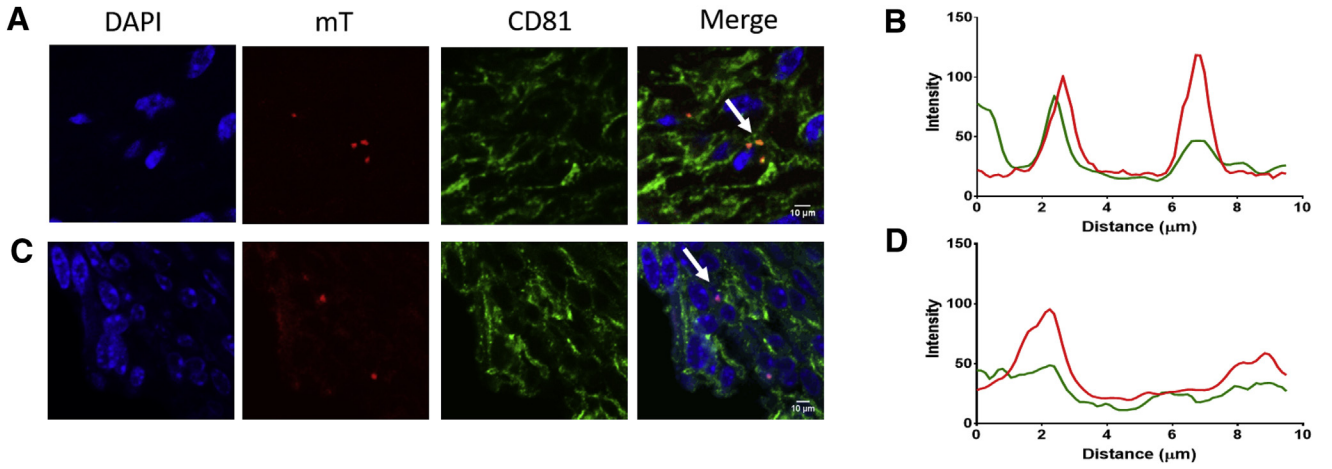
Determination of exosome size and concentration

The size distribution and concentration of exosomes were determined using the Nanosight NS300 (Malvern Instruments, Worcestershire, UK) as described previously.[28] Our previous studies have shown that fetal exosomes can cross to the maternal compartment.[29] To determine the percentage of fetal exosomes in maternal plasma, we divided the concentration of fetal exosomes in maternal plasma (immunoprecipitated using mT) by the total concentration of exosomes in maternal plasma.

Determination of exosome shape using cryo—electron microscopy

Cryo—electron microscopy (Cryo-EM) was used to determine the shape of

FIGURE 3
Fetal exosomes express CD81 and colocalized with maternal cells



A—D, Fluorescence microscopy was used to colocalize fetal exosomes expressing membrane-targeted tdtomato (mT) (red) with exosome marker CD81 (green) in maternal cervix (**A**) and uterus (**C**). Colocalization line graphs were created to illustrate overlap of mT and CD81 in cervix (**B**) and uterus (**D**). White arrows indicate areas of colocalization.

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exosomes from total maternal plasma and after mT and mG IP. Prepared exosome suspension in PBS was pipetted onto a copper grid with quantifoil support film (QUANTIFOIL, Großlöbichau, Germany). The grid was vitrified (to preserve exosomes in their native state) and then placed in a Gatan 626 specimen holder (Gatan, Pleasanton, CA), within a JEOL 2100 microscope for transmission electron microscopy (JEOL, Osaka, Japan). The samples were imaged at 15,000−30,000 magnification with a 200 kV electron beam from a LaB6 emission source, and images were recorded on a Gatan US4000 CCD camera.

Determination of exosome markers

To determine exosome markers from the IP for mT or mG, exosomes eluted from beads were analyzed using Exo-Check Exosome Antibody Array (System Biosciences) following manufacturer instructions. Blots were visualized using a ChemiDoc Imaging System (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

Nanoparticle tracking analysis of fetal and total exosomes were determined from n = 4 mice. A Student *t* test was performed, and $P < 0.05$ was considered significant.

Results

Validation of mT exosomes in mouse fetal and maternal tissues

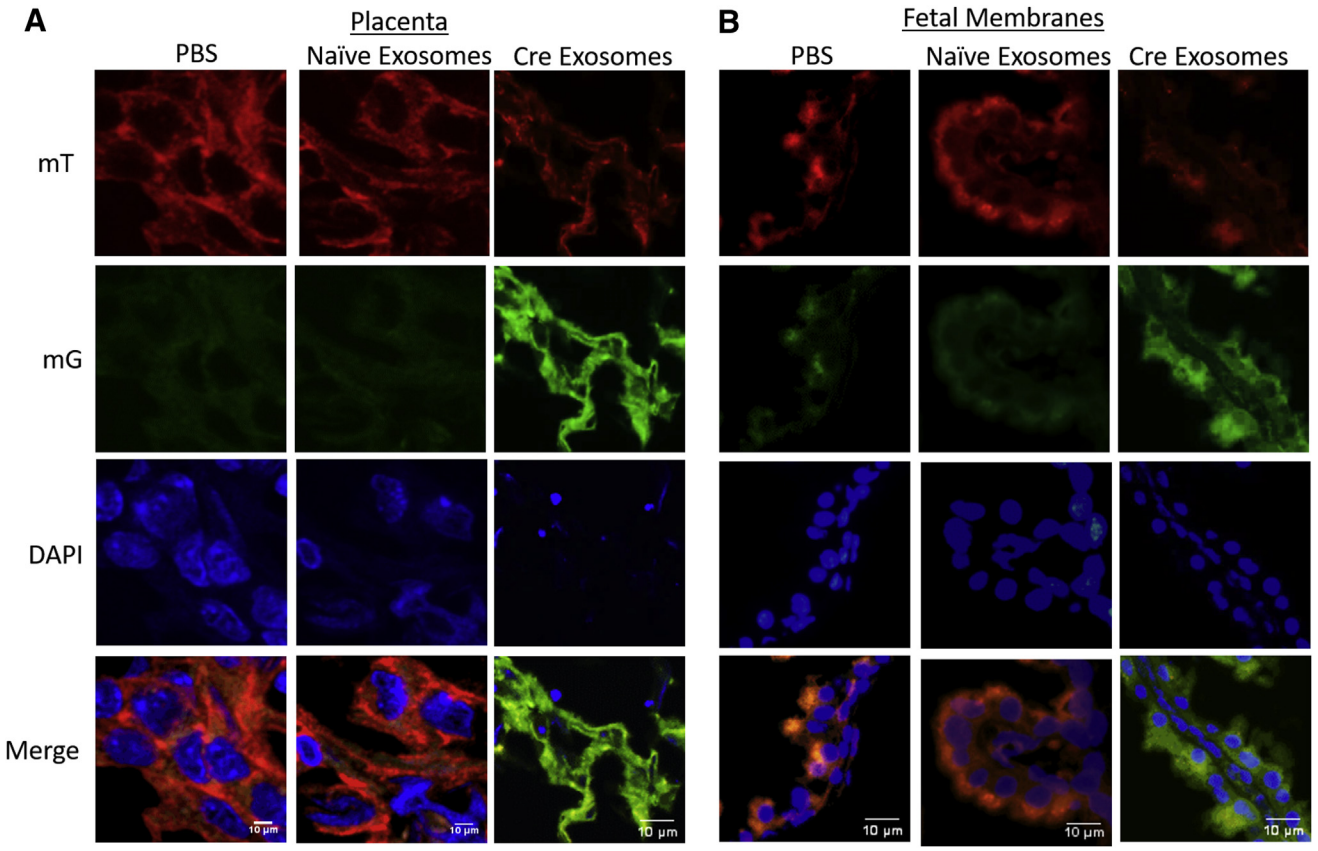
Expression of mT in fetal tissues

An animal model of pregnancy in which only fetal cells and fetal cell−derived exosomes expressed a specific marker, was developed to allow us to isolate either fetal cells or exosomes on the maternal side to further study fetal specific signaling. Wild-type (WT) females were mated with mT/mG homozygous males (Figure 1A and 1B) to show that fetal tissues heterozygous for the mT/mG allele still express mT in the absence of Cre. On E16, the placenta (Figure 2A) and fetal membranes (Figure 2B) showed expression of mT (red) with minimal expression of mG (green). The minimal expression of mG in the absence of Cre was expected due to potential low levels of recombination.

Maternal plasma exosomes express mT

Previous studies in our laboratory have shown that fluorescently labeled exosomes injected intra-amniotically can be isolated from maternal circulation, suggesting potential trafficking of exosomes from the fetal side.[29] However, these are exogenously administered exosomes and not produced by the fetus. After determining mT expression in fetal tissues, we tested whether exosomes released from these tissues carry mT and whether they can be isolated from maternal plasma samples. For this, exosomes isolated from maternal plasma were analyzed using bead-coupled flow cytometry. After total exosome isolation, anti-mT was used to pull down fetal exosomes that were subsequently analyzed using flow cytometry (Figure 2C). We found that 16.6% of the beads were positive for mT-expressing exosomes (mT+). Fetal (precipitated from beads) and total exosomes were further analyzed for size (Figure 2D) and concentration (Figure 2E) using nanoparticle tracking analysis (NTA). As expected, the total maternal plasma exosome concentration was significantly greater ($1.62 \times 10^{10} \pm 1.26 \times 10^{10}$) than the fetal-specific exosome concentration ($5.66 \times 10^{9} \pm 8.81 \times 10^{8}$, $P = 0.01$), which was about 35% of the total exosomes, whereas exosome size between fetal (166.4 ± 9.5) and total (147.3 ± 16.6) exosomes did not differ significantly.

FIGURE 4
Exosomes can cross from the maternal side to the fetal compartment



A and **B**, To determine maternal-to-fetal communication via exosomes, we performed maternal intraperitoneal injections of cyclic recombinase (Cre)–enriched exosomes. Crossing of Cre-enriched exosomes through the placental barrier and their uptake by fetal cells are expected to test the functional changes that an exosome can produce. This also confirms maternal-to-fetal trafficking of exosomes. Phosphate-buffered saline and naive exosome (Cre-negative) injected mice had dominant expression of membrane-targeted tdtomato (mT) in the placenta (**A**) and fetal membranes (**B**), whereas Cre exosome injected mice had higher expression of membrane-targeted enhanced green fluorescent protein (mG) than mT. Scale bars, 10 μm.

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mT is expressed in WT maternal uterus and cervix

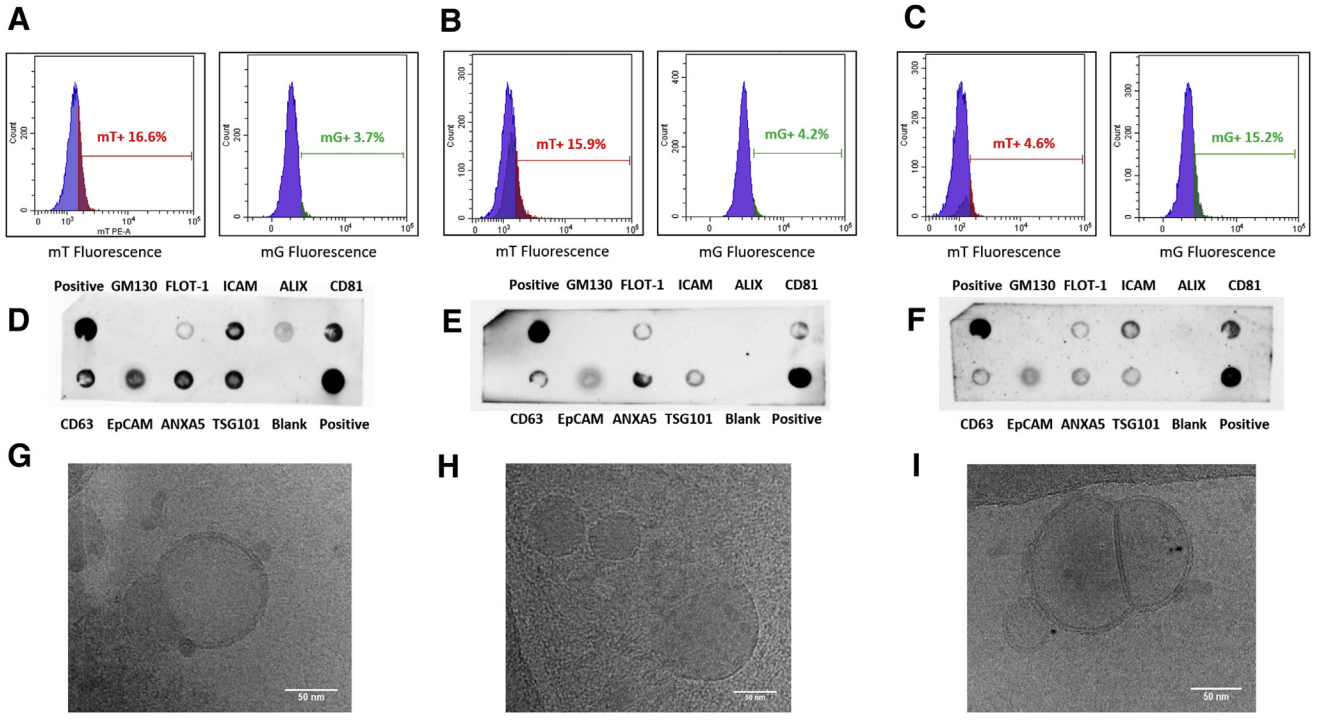
After determining the systemic spread of exosomes from the fetal to the maternal side, we further localized their trafficking to specific uterine tissues. Confocal microscopy localized fetal exosomes in the maternal uterus and cervix. Tissues stained for exosome marker CD81 were colocalized with mT in cervix (Figure 3A) and uterus (Figure 3C). Line graphs were generated to graphically represent mT colocalization with CD81 (Figure 3B and D). As shown in Figure 3A–D, mT expression in the maternal cervix and uterus suggested trafficking of fetal exosomes to maternal tissues.

Cre-enriched exosomes injected maternally traffic to fetal tissues and induce functional changes

After showing mT expression in fetal cells and trafficking of mT+ exosomes and mT+ cells to the maternal side, we used this animal model to test the following: (1) trafficking of exosomes from the maternal side by crossing the placental barrier; and (2) exosomes from the maternal side causing functional changes in fetal tissues. For this, Cre-enriched exosomes were generated using EXPLOR technology to introduce Cre as an exosome cargo to cells containing the gene construct (Figure 1A, 1B), as Cre is expected to cleave mT, leading to mG expression. The EXPLOR method generated Cre-enriched exosomes for further use.[31]

To determine exosome-mediated maternal-to-fetal communication and functional changes, intraperitoneal injection of Cre-enriched exosomes, naive exosomes (Cre-negative), or phosphate-buffered saline (PBS) on the maternal side on E13 was performed. Uptake of Cre-enriched exosomes by fetal cells can cause removal of mT cassette and lead to the expression of mG (mG+) (Figure 1A). On E16, placenta (Figure 4A) and fetal membranes (Figure 4B) were examined for mT and mG expression, as detected by confocal microscopy. As shown in Figure 5, injection of Cre-enriched exosomes

FIGURE 5
Cre exosome functional changes are reflected in maternal plasma exosomes



A—C, Maternal plasma exosomes were isolated and analyzed for membrane-targeted tdtomato (mT) and membrane-targeted enhanced green fluorescent protein (mG) expression in phosphate-buffered saline (**A**), naive exosome (**B**) and Cre exosome (**C**) using bead-coupled flow cytometry. **D—F,** Exosomes were removed from beads, and total plasma exosomes (**D**), mT-expressing exosomes (**E**), and mG-expressing exosomes (**F**) were analyzed for exosome markers using dot blots. **G—I,** Total plasma exosomes (**G**), mT-expressing exosomes (**H**), and mG-expressing exosomes (**I**) were visualized using cryo—electron microscopy. Scale bar, 50 nm.

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resulted in replacement of mT with mG in both placenta (Figure 4A) and fetal membranes (Figure 4B), whereas PBS and naive exosome injected mice showed dominant expression of mT compared to mG in both placenta and fetal membranes. This suggests that maternal exosomes can traffic to the fetal side and cause functional changes.

mG+ exosomes released from Cre altered fetal cells traffic back to maternal plasma

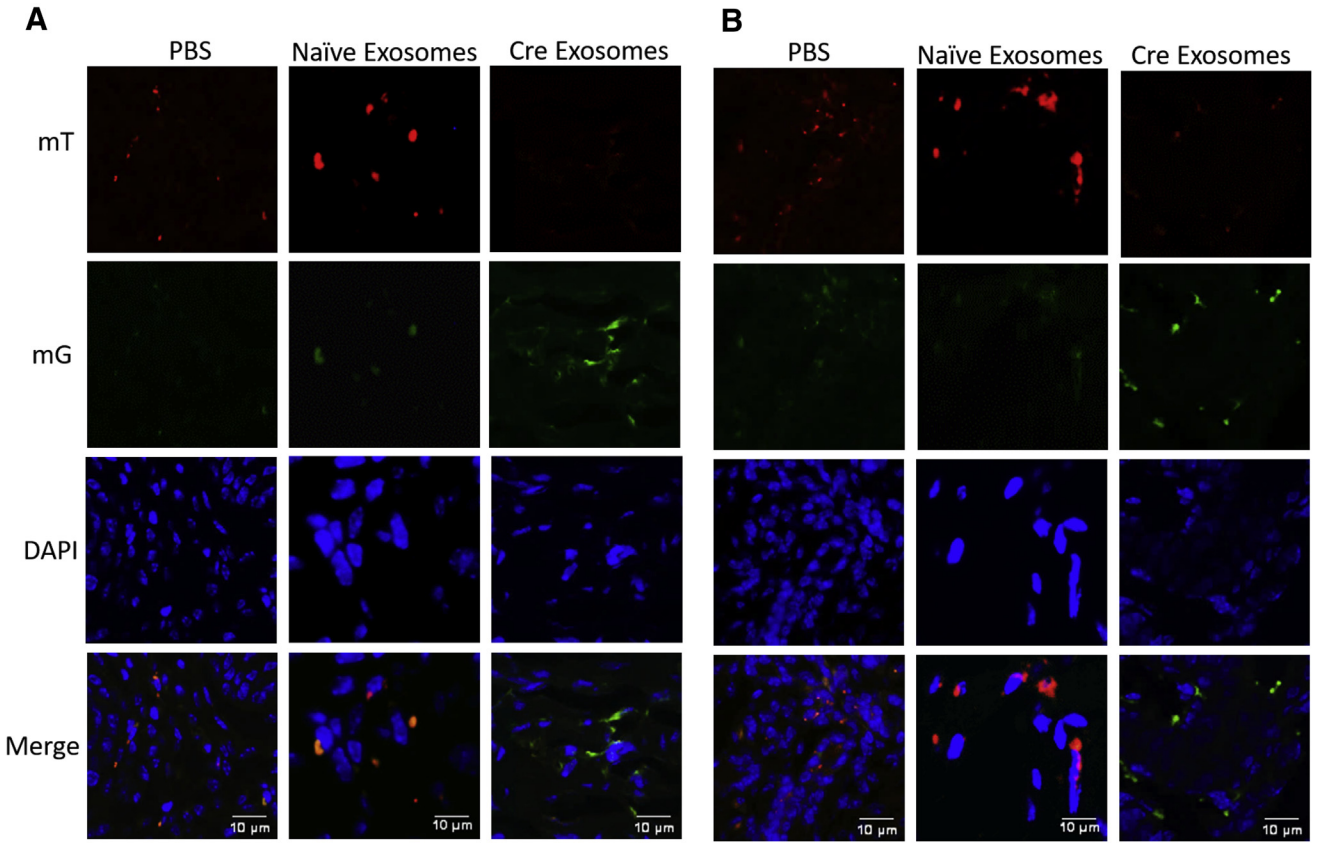
To determine whether Cre-mediated expression changes were reflected on the maternal side (trafficking of fetal exosomes to the maternal side), we isolated exosomes from maternal plasma and performed bead-coupled flow cytometry on mT and mG expressing exosomes from Cre (Figure 5A), naive exosome (Figure 5B), and PBS (Figure 5C) injected mice. As shown in Figure 5C, Cre exosome injection produced an increase in mG+ (15.2%) beads in maternal plasma compared to mT+ (4.6%). PBS (16.6%) (Figure 5A) and naive (15.9%) (Figure 5B) exosome injected mice had higher mT+ beads than mG. These data suggest that Cre exosome injection produced functional changes in fetal tissues, which were reflected in the maternal plasma exosome composition.

To confirm that we were isolating exosomes with expected size, shape, and markers, exosome marker dot blots (Figure 5D—F) and cryo-electron microscopy (cryo-EM, Figure 5G—I) were performed on maternal plasma exosomes isolated from Cre exosome, naive exosome, and PBS injected animals. Irrespective of IP for either mT or mG, exosomes had a spherical morphology, consistent with prior reports,[36—38] and expressed exosome markers ICAM, CD81, CD63, EpCAM, ANXA5, and TSG101. GM130, a Golgi vesicle protein marker, was absent in all of our exosome preparations, confirming the lack of any other contaminant extracellular vesicles such as other microvesicles or apoptotic bodies.

Fetal-derived exosomes traffic to maternal reproductive tissues after Cre-induced functional changes

To further confirm the phenomenon that fetal exosomes can traffic to maternal tissues after injection with Cre exosomes, we performed confocal microscopy to detect colocalized fetal exosomes expressing mG in maternal cervix (Figure 6A) and uterus (Figure 6B). After injection with Cre exosomes, mT decreased and mG

FIGURE 6
Cyclic recombinase (Cre)—induced changes are reflected in exosomes in maternal tissues



A and **B**, Maternal tissues were collected and analyzed using fluorescence microscopy for membrane-targeted tdtomato (mT) to membrane-targeted enhanced green fluorescent protein (mG) expression changes after Cre exosome injection. Phosphate-buffered saline and naive exosome (Cre-negative) injected mice displayed expression of mT in the cervix (**A**) and uterus (**B**) Cre exosome injected mice had higher expression of mG than mT. Scale bars, 10 μm.

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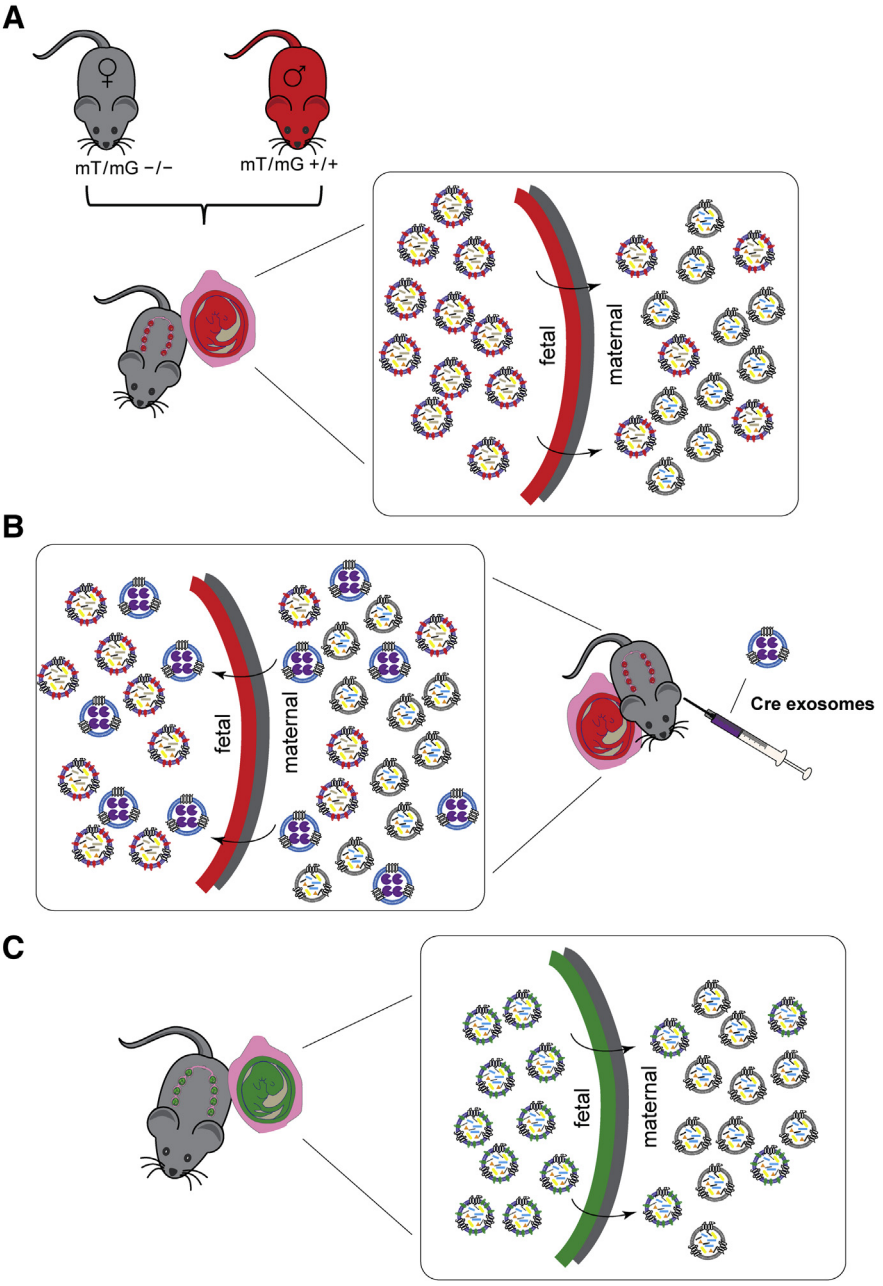
expression increased in both cervix and uterus. As expected, PBS and naive exosome injected cervixes and uteri had greater mT expression than mG. The shift of mT to mG-expressing exosomes in maternal plasma further supports fetal-to-maternal trafficking of exosomes. It is also intriguing that exosomal delivery of a maternal cargo causes functional changes in the fetus that can still be monitored on the maternal side.

Comment

Constant communication between the mother and her fetus is essential for the maintenance of pregnancy and to determine timing of labor and delivery. Endocrine and immune factors are well reported communication mechanisms[39–43]; however, unique characteristics of exosomes, including their size, structural proteins and lipids, and their ability to carry cargo that would otherwise be susceptible to degradation, to distant destinations in the body[44,45] makes them ideal candidates as signals between feto-maternal tissues during pregnancy. Although placental exosomes can be isolated from maternal plasma during human pregnancies, because of the impracticality of functional studies in humans, animal models are essential to understand exosomal feto-maternal communication during pregnancy and parturition.[46–49] Although mouse parturition differs from that of humans with respect to endocrine signaling at term,[50–52] similarities in paracrine signaling mechanisms (ie, exosomes) make the mouse a viable model to study exosome-mediated feto-maternal communication.[24,29] Conditional gene targeting using site-specific recombinase systems, like the Cre/lox system, is primarily used to study tissue-specific gene knockdown by linking the Cre/loxP genes with a tissue-specific protein to allow for conditional targeting of a protein of interest.[53] Injection of HEK293T cell–derived exosomes crossed the placental barrier and did not cause any adverse immune reaction on either the maternal or the fetal side in our mouse model. Excision of mT by Cre, leading to mG expression in fetal tissues and their exosomes, promises to be a novel approach for delivery of desired cargo that can reach fetal tissues, such as therapeutic agents for treating the fetus during adverse pregnancies. Tropism of exosome

FIGURE 7
Illustration of feto-maternal communication via exosomes. Our model uses a cyclic recombinase (Cre)–dependent dual fluorescent reported mouse to monitor fetal exosomes in maternal circulation



A, Female wild-type (WT) mice were mated with males homozygous for the double fluorescent Cre reporter construct (mT/mG) so that all fetal tissue express membrane-targeted tdtomato (mT)/membrane-targeted enhanced green fluorescent protein (mG) whereas maternal tissues do not. Fetal exosomes carrying mT can migrate to the maternal compartment and can be localized in maternal circulation and reproductive tissues. **B** and **C**, Cre-enriched exosomes injected intraperitoneally on the maternal side can traffic to the fetal compartment, where they cause functional changes when the Cre in exosomes release mT and have mG expression. The mT-to-mG change is reflected in fetal tissues as well as in maternal compartments. Fetal exosomes trafficking to the maternal side carrying mG can be localized in maternal circulation and reproductive tissues.

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trafficking, irrespective of direction (ie, fetal to maternal or maternal to fetal) was not the focus of this study.

Fetal-specific membrane-targeted fluorescent proteins, mT, or mG, expressing exosomes were isolated in maternal plasma (Figure 7A and 7C). The presence of mG expressing exosomes also confirmed the functional changes produced by a signal from the maternal side (Cre-enriched exosomes) migrating to the fetal tissues (Figure 7B). The detection of fetal exosomes in maternal plasma enhances its biomarker potential for use during pregnancies using minimally invasive liquid biopsy specimens. Although this model allows for the selection of fetal vs maternal exosomes in maternal plasma, the origin of fetal exosomes may be fetal membranes, placenta, or the fetus itself.

We acknowledge that the Cre-enriched exosomes also carry other cellular cargo. Although the focus of this study was on Cre functional effects, it must be noted that other effects may occur due to additional cargo contained in the exosomes. Injection of naive exosomes did not show any Cre-specific effects, as expected, or any other adverse outcomes, confirming that the observed effects are indeed produced by Cre. This pregnant mouse model allows longitudinal assessment of fetal development, fetal response to maternal exposures, and feto-maternal exchange of various materials via exosomes, which can be made without sacrificing the animals. We acknowledge that endocrine regulation[54,55] and multifetal pregnancy in the mouse are different compared to humans. Despite these differences, fetal membrane senescence[32,56,57] and paracrine signaling via exosomes[24,29,58] are similar mechanisms associated with labor in humans and mice. Efficiency of the mT to mG transition by Cre exosomes was not 100%, as evidenced by mT expression in fetal tissues before and after Cre-enriched exosome injection. Better functional efficiency will most likely be seen using intra-amniotic injections, which are more invasive and less ideal if using this approach to mimic potential treatments for women at risk for adverse pregnancy outcomes.

The photostability of both fluorescent reporter proteins and the membrane-targeting aspect of this transgenic mouse model will pave the way for future research investigating the role of exosomes in normal and adverse complications of pregnancy. Being able to selectively analyze fetal as opposed to maternal exosomes allows the opportunity to further our understanding of feto-maternal communication during pregnancy and labor that involves multiple obstacles, including the uniqueness of the maternal–fetal system of managing 2 individuals. This exosome mouse model allows advancement of progressive fetal research without performing invasive procedures or sacrificing the animals. ■

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Author Contributions

S.S.-M. conceived and designed experiments, conducted the study, performed all experimental aspects of the project, analyzed and interpreted data, and prepared the manuscript. R.M. helped with experimental design, data analysis and interpretation, provided funds and other resources, and preparation of manuscript. K.C. and C.C. provided cyclic recombinase (Cre) and naive exosomes and edited the manuscript.

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Author and article information

From the Division of Maternal-Fetal Medicine & Perinatal Research (Drs Sheller-Miller and Menon), Department of Obstetrics & Gynecology, The University of Texas Medical Branch, Galveston, TX; ILIAS Biologics, Inc. (Drs Choi and Choi), Daejeon, Republic of Korea; Department of Bio and Brain Engineering, KAIST (Dr C. Choi), Daejeon, Republic of Korea.

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Corresponding author: Ramkumar Menon, MS, PhD. ra2menon@utmb.edu