THE ROLE OF THE FETAL MEMBRANES IN PREGNANCY AND BIRTH

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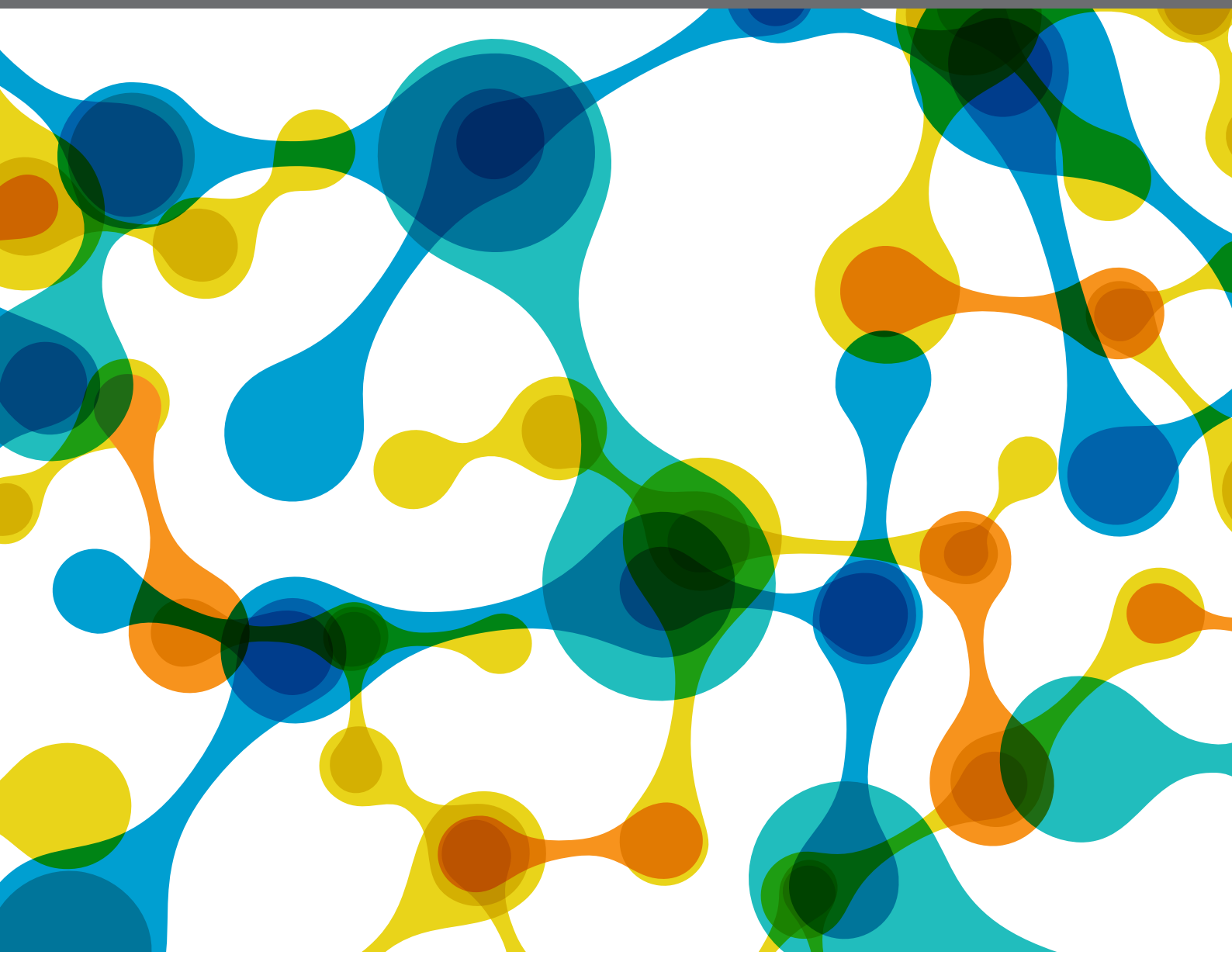

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THE ROLE OF THE FETAL MEMBRANES IN PREGNANCY AND BIRTH

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EDITORIAL
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Editorial: The Role of the Fetal Membranes in Pregnancy and Birth

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Keywords: fetal membranes, PPROM, preterm birth, amniochorion, pregnancy

Editorial on the Research Topic

The Role of the Fetal Membranes in Pregnancy and Birth

Fetal membranes or amniochorionic membranes are one of the most intriguing tissues in the intrauterine cavity that are essential for the protection of the fetus, maintenance of pregnancy, and as a signaler to initiate parturition (Menon et al., 2018). However, the structure, biology, life cycle, and functions of the fetal membranes are unclear to many in the field of reproductive biology. Hence, several knowledge gaps exist that have hindered the advancement of pregnancy research as well as designing strategies to mitigate the dysfunction of this tissue, frequently associated with adverse pregnancy outcomes. This is partly because fetal membranes are often considered a mere appendage of the placenta, or even dead tissue at fetal delivery. Consequently, fetal membrane research has often been misguided, ignored, and vastly unfunded in reproductive biology and medicine (Menon and Moore, 2020). Ironically, successes in branches of medicine outside of reproductive biology, like regenerative medicine utilizing the stem cell-like properties, survivability past placental delivery, and transitional features of amniochorion cells have generated vigorous renewed interest to further understand this tissue (Martin et al., 2019).

Developmentally, fetal membranes are composed of two separate layers of tissues (**Figure 1**); (1) a single cell epithelial layer of amnion that forms the innermost lining of the uterine cavity, bathed in amniotic fluid and in constant touch with the fetus, and (2) the outer chorion trophoblast layer that forms the feto-maternal interface barrier by lining the maternal decidua (Menon et al., 2018). This interface is distinct from the placental-decidual interface and often not studied. The amniochorion layers are connected through a collagen rich extracellular matrix that contains amnion and chorion mesenchymal cells. Amnion and chorion layers start their growth and development at the time of implantation from distinct lineages. The amnion is derived from extraembryonic somatic mesoderm and epiblast-derived amnioblasts whereas the chorion is formed by extraembryonic mesoderm and trophoblasts (Luckett, 1978; Rasweiler, 1990; Carter, 2016). The amnion and the chorion fuse to become a single unit structure around the late 1st or early 2nd trimester to shape up the intrauterine cavity (Ulm et al., 1999). Amniochorion provides mechanical, immune, endocrine, transport, and antimicrobial functions during pregnancy. Fetal membranes age as gestation progresses and it is correlated with fetal growth and development (Menon and Richardson, 2017; Menon et al., 2020). As longevity of the membranes approaches its limit at term, membranes show aging pathology (dysfunctions) highlighted by inflammation. Inflammatory mediators from aging fetal membranes are among the key fetal biological signals to initiate parturition. Thus, fetal membranes help to maintain

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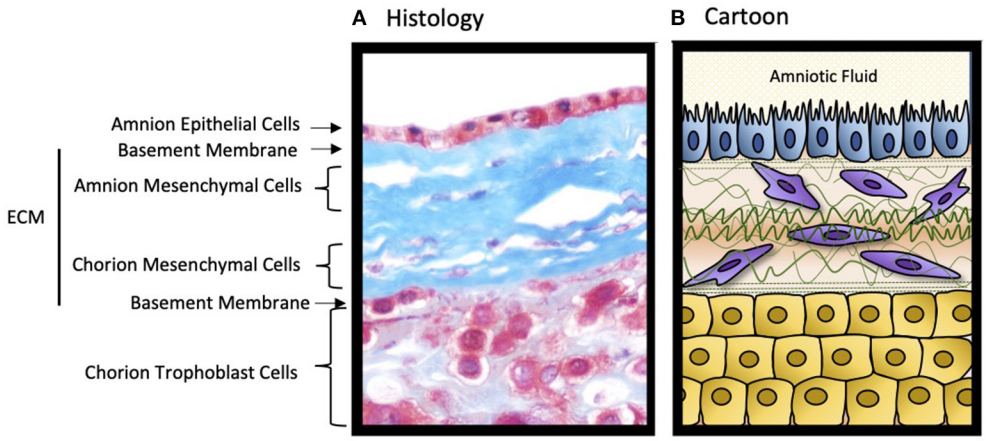

FIGURE 1 | The structure of the human fetal membranes. **(A)** Microscopic cross section of the fetal membrane tissues stained with Masson's trichrome. **(B)** Cartoon representation of the histological image. The decidua (not shown) is fused to the chorion trophoblast cell layer forming the feto-maternal interface. ECM, extracellular matrix. The illustration has been created by Dr. Lauren Richardson.

pregnancy as well as promote parturition (Menon et al., 2020). Compromising the cellular and matrix architecture of the membranes can be detrimental contributing to various adverse pregnancy outcomes, particularly preterm premature rupture of the membranes (pPROM) and spontaneous preterm birth (PTB). Rupture of the membranes at term is a physiologic requirement for feto-placental delivery; however, pPROM is associated with ∼40% of all PTB. Currently, there are no diagnostic indicators for these conditions and early delivery is the best available strategy to avoid complications to the developing fetus. A better understanding of fetal membrane functions *in utero*, mechanisms underlying its longevity, and cellular biological properties are essential to determine the pathological complications contributing to its dysfunctions and adverse pregnancy outcomes.

The challenges to understand fetal membrane functions and dysfunctions are substantial. A major issue is the impracticality of obtaining fetal membrane tissues during pregnancy unless delivered preterm. In addition, pregnancy complications associated with preterm deliveries can confound membrane studies. Animal models often do not mimic human fetal membranes. These circumstances impede research and limit the translational impact of work in the field. To help addressing the needs of interested scientists, the Fetal Membrane Society (FMS https://www.fetalmembranesociety.org/) was formed in 2019. This Research Topic has been assembled in response to encouragement by the FMS to highlight the latest data and chart future prospects in fetal membrane research.

The collection includes 16 articles covering the basic, functional, and translational aspects of fetal membrane biology to show how these tissues contribute to pregnancy and pregnancy associated pathologies. Three manuscripts discuss the *genomics, epigenomics, and proteomics* of fetal membranes. Cunningham et al. and Zakar and Paul reviewed articles on fetal membrane genome, exome, methylome, and epigenome and displayed differences in the expression of various functional molecules in normal and abnormal pregnancies. Proteomics analysis of fetal membranes from normal and preterm pregnancies was discussed by Pan et al. showing how inflammatory pathways and collagen metabolisms are impacted in preterm births either with or without pPROM. Fetal membranes are a rich source of various *endocrine mediators and their receptors*. The roles of fetal membrane-derived eicosanoids and exosomes in human parturition are discussed by Mosaad et al. Further, endocrine mediators support cellular and matrix homeostasis. Kang Sun's group has contributed immensely to the knowledge of cortisol regeneration in fetal membrane matrix (mesenchymal) cells by 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1). A review on this topic is provided by Wang et al. which discusses the physiologic and pathologic contributions of 11β-HSD1 in fetal membranes and its contributions to preterm and term parturitions. Another key mediator that maintains fetal membrane homeostasis during pregnancy is progesterone. Marinello et al. describes how amnion membrane matrix mesenchymal cells use cell membrane-associated progesterone receptors in providing a progesterone dependent anti-inflammatory environment during pregnancy.

Like all other tissues, fetal membranes start aging *in utero* and aging progresses during gestation. Premature *senescence of the fetal membranes* leading to dysfunction is one of the reasons underlying pPROM and PTB. The review by Polettini and Guimarães da Silva focuses on the telomere-dependent aging of fetal membranes and its association with telomerase dynamics.

Fetal membrane pathologies are often associated with *inflammation.* One of the classic signs that describe the severity of adverse pregnancy outcomes and a factor often used to design management strategies of preterm neonates is based on the histologic assessment of fetal membrane inflammation. Infiltration by neutrophils, termed histologic chorioamnionitis, is an indicator of the severity of inflammation

in the intrauterine cavity. Compromising immune defense is one of the mechanisms causing fetal membrane inflammation, immune cell infiltration, and membrane dysfunctions leading to chorioamnionitis-associated preterm birth or pPROM. *Exogenous risk exposures* and their interactions with endogenous factors like the microbiome often amplify pregnancy risk. An article about maternal environmental pollutant exposures that can weaken innate immune defense is discussed by Harris et al. In this review, authors report risk modification by an interaction between the environment and pathogenic vaginal colonizers such as Group B streptococcus. Wheeler and Oyen review data related to the interesting hypothesis that fetal membranes may actually behave as an inflated balloon, and pressure changes during severe weather events can promote pPROM. Inflammation and inflammatory cytokines are well-studied in the fetal membranes. A report by Pardon et al. in this Research Topic and multiple reports by Vincent Sapin and Loïc Blancho's group have provided novel ideas to this area by discussing the roles and contributions of *damage associated molecular patterns* (DAMPs) and their receptors. By analyzing DAMPs and their receptors on *maternal* biological samples and exosomes, Sapin's group report a lack of association between these markers and pPROM. As reported, high abundance of DAMPs in *fetal* membranes and compartments is suggestive of fetal contributions to preterm birth and pPROM.

An understudied area of fetal membrane research is its *growth, remodeling, and repair*. A better understanding of these mechanisms can improve outcomes of fetoscopy and fetal surgeries as these procedures often contribute to membrane rupture and preterm delivery. Based on their own reported findings, Mogami and Word summarize how epithelial mesenchymal transition (EMT) of amnion epithelial cells can be a mechanism in fetal membranes that can heal membrane wounds. Approaches to enhance this process are also discussed. Multiple factors have been described to promote EMT in various cells. One of them is IL-6, a cytokine produced in high abundance during fetal membrane dysfunctions. IL-6 has been considered one of the cytokines contributing to preterm birth and pPROM as its levels are very high in all pregnancy associated biological fluids and tissues. A basic science report is included in this Research Topic by Omere et al. where they studied IL-6's effect on amnion epithelial cells. IL-6 lacks cell fate determining properties even at levels seen during pPROM or PTB and it does not appear to promote EMT in fetal membrane cells.

Lack of biomarkers indicative of membrane dysfunction hampers clinical management of membrane-associated complications such as pPROM and spontaneous preterm birth. An innovative approach using MRI has been introduced recently by Wang et al. to determine membrane pathologies. The potential of this approach as an *imaging-based biomarker* to test membrane function is reviewed by Qi et al. In their report, Omere et al. also describe the role of IL-6 as a potential biomarker associated with adverse events, although IL-6 does not seem to have any specific impact on fetal membrane cells.

Fetal membrane research is notoriously obstructed by a lack of specialized techniques and tools. Richardson et al. points out how innovative approaches like fetal membrane *organ on a chip* (OOC) can be used to replace traditional 2D cell cultures, transwells, and organ explant models to study fetal membrane biology and function. In their review, three phases of fetal membrane OOCs development have been outlined and its advantages and disadvantages are discussed. Without a doubt, the *in vitro* reconstruction of fetal membrane tissues is an exciting futuristic approach with great potential.

In summary, the Research Topic "The Role of the Fetal Membranes in Pregnancy and Birth" provides a comprehensive overview of fetal membrane biology, endocrine, mechanical, immune functions, normal and risk-associated changes to the membranes, development of biomarkers to diagnose membrane functions or dysfunctions, and tools to study fetal membranes. The collection of papers, under the auspices of the Fetal Membrane Society, will certainly promote fetal membrane biology to take its deserved place of prominence in perinatal science.

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RM, ML, and TZ contributed to this editorial. All authors contributed to the article and approved the submitted version.

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REVIEW
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Cortisol Regeneration in the Fetal Membranes, A Coincidental or Requisite Event in Human Parturition?

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The fetal membranes are equipped with high capacity of cortisol regeneration through the reductase activity of 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1). The expression of 11β-HSD1 in the fetal membranes is under the feedforward induction by cortisol, which is potentiated by proinflammatory cytokines. As a result, the abundance of 11β-HSD1 increases with gestational age and furthermore at parturition with an escalation of cortisol concentration in the fetal membranes. Accumulated cortisol takes parts in a number of crucial events pertinent to the onset of labor in the fetal membranes, including extracellular matrix (ECM) remodeling and stimulation of prostaglandin output. Cortisol remodels the ECM through multiple approaches including induction of collagen I, III, and IV degradation, as well as inhibition of their cross-linking. These effects of cortisol are executed through activation of the autophagy, proteasome, and matrix metalloprotease 7 pathways, as well as inhibition of the expression of cross-linking enzyme lysyl oxidase in mesenchymal cells of the membranes. With regard to prostaglandin output, cortisol not only increases prostaglandin E2 and F2α syntheses through induction of their synthesizing enzymes such as cytosolic phospholipase A2, cyclooxygenase 2, and carbonyl reductase 1 in the amnion, but also decreases their degradation through inhibition of their metabolizing enzyme 15-hydroxyprostaglandin dehydrogenase in the chorion. Taking all together, data accumulated so far denote that the feedforward cortisol regeneration by 11β-HSD1 in the fetal membranes is a requisite event in the onset of parturition, and the effects of cortisol on prostaglandin synthesis and ECM remodeling may be enhanced by proinflammatory cytokines in chorioamnionitis.

Keywords: 11β-HSD1, collagen, prostaglandins, glucocorticoids, fetal membranes

INTRODUCTION

Glucocorticoids are essential for life, and it regulates a variety of important cardiovascular, metabolic, and immunologic functions in the maintenance of homeostasis (Schmid et al., 1995; Beato and Klug, 2000; Rhen and Cidlowski, 2005). Cortisol is the most important endogenous glucocorticoid in humans. The *de novo* synthesis of cortisol from cholesterol takes place primarily

in the zona fasciculata of the adrenal cortex (Miller and Auchus, 2011). After secretion into the circulation, most of cortisol is bound by corticosteroid-binding protein (CBG) and to a lesser extent by albumin (Bae and Kratzsch, 2015; Meyer et al., 2016). There is approximately only 5 to 10% of cortisol that remains free in the circulation, which is important for the actions of cortisol as only the free fraction of cortisol is biologically active (Lewis et al., 2005). In compensation, glucocorticoid target organs develop a way to enhance cortisol concentrations within the cells through regeneration of cortisol by 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) (Chapman et al., 1997; Tomlinson et al., 2004; Chapman et al., 2013; Morgan et al., 2014). 11β-HSD1 is a microsomal reductase catalyzing the regeneration of cortisol from biologically inactive 17α-hydroxy-11-dehydrocorticosterone (cortisone), which derives mostly from the oxidase action of 11β-HSD2 in the mineralocorticoid target organs (**Figure 1**; Tannin et al., 1991; Albiston et al., 1994; Chapman et al., 2013). 11β-HSD2 is a counterpart enzyme of 11β-HSD1 and functions in an opposite way to 11β-HSD1 converting biologically active cortisol to inactive cortisone (**Figure 1**). Because 11β-HSD2 does not metabolize aldosterone, 11β-HSD2 is utilized by the mineralocorticoid target organs as a pre-receptor gate to ensure the indiscriminating mineralocorticoid receptor being occupied only by aldosterone but not by cortisol (White et al., 1997a,b,c). This differential expression pattern of 11β-HSD1 and 11β-HSD2 in glucocorticoid and mineralocorticoid target organs is developed perfectly to ensure the efficiency of cortisol's actions and the specificity of aldosterone's actions in their respective target organs.

In pregnancy, the placenta is responsible for nourishing and protecting the fetus as well as maintaining pregnancy by producing a plethora of hormones and immune factors. Attached to the edge of the discoid placenta is the atrophied chorionic villi, also known as the smooth chorion or chorion leave, which fuses with the amniotic membrane extended from the fetal surface of the placenta, and together they form the reflected fetal membranes (Leiser and Kaufmann, 1994; Ferner and Mess, 2011). The fetal membranes not only enclose the fetus bathed in the amniotic fluid but also become a source of initiating signals for parturition toward the end of gestation (Okazaki et al., 1981; Myatt and Sun, 2010; Menon, 2016; Wang et al., 2018; Menon and Moore, 2020). Like the specific distribution of 11β-HSD1 and 11β-HSD2 in glucocorticoid and mineralocorticoid target organs, the distribution of 11β-HSD1 and 11β-HSD2 in the placenta and fetal membranes also adopts a unique tissue-specific pattern (Sun et al., 1997; Yang et al., 2016). Although the placenta is not a typical mineralocorticoid target organ, it boasts abundant 11β-HSD2 but scarce 11β-HSD1 (Albiston et al., 1994; Sun et al., 1997; Yang et al., 2016). It is known that 11β-HSD2 in the placenta functions as a glucocorticoid barrier by inactivating maternal cortisol to cortisone so that the fetus can be protected from the growth-restricting effects of excessive maternal glucocorticoids (Osinski, 1960; Burton and Waddell, 1999; Drake et al., 2007). This function of 11β-HSD2 in the placenta is substantiated by its distinct distribution in the syncytiotrophoblast, the outmost layer of placental villi that immerse directly in the maternal blood (Krozowski et al., 1995; Ni et al., 2009; Li et al., 2011, 2013; Zhang et al., 2015; Zuo et al., 2017). In contrast to the placenta, the fetal membranes express abundant 11β-HSD1 with barely detectable 11β-HSD2 (Sun et al., 1997), which can utilize cortisone derived from both maternal mineralocorticoid organs and the placenta to regenerate cortisol (**Figure 1**; Murphy, 1977, 1979). The expression of 11β-HSD1 in the fetal membranes increases with gestational age and further increases in parturition with its abundance atop all fetal tissues by the end of gestation (Murphy, 1977, 1981; Alfaidy et al., 2003). This cortisol-regenerating capacity of the fetal membranes is even regarded as a supplemental extra-adrenal source of glucocorticoids in pregnancy (Murphy, 1977, 1981; Tanswell et al., 1977). Why should the fetal membranes be equipped with such a unique cortisol-regenerating capacity in pregnancy? Given that the smooth chorion is actually atrophied chorion villi, it is plausible to question whether this cortisol regeneration activity of the fetal membranes is merely an irrelevant by-product of pregnancy or a finely mapped-out event, which is absolutely required toward the end of gestation. In this review article, we will try to answer these questions by summarizing data from our laboratory as well as others.

FEEDFORWARD CORTISOL REGENERATION IN THE FETAL MEMBRANES

The fetal membrane components differ in different species (Mess et al., 2003). Human fetal membranes comprised the amnion and smooth chorion. The smooth chorion is the outer layer that connects the maternal decidua and can be subdivided into a trophoblast layer and a connective tissue layer adjacent to the amnion. The amnion is composed of a single layer of amnion epithelial cells sitting on a basement membrane and a tough compact layer that contains abundant interstitial fibers and fibroblasts (Bourne, 1960; Parry and Strauss, 3rd., 1998; Wang et al., 2018). Studies of 11β-HSD1 in the fetal membranes of other species are sparse. However, 11β-HSD1 has been localized to the placenta in a number of species including rat, sheep, and baboon (Burton and Waddell, 1994; Pepe et al., 1996; Yang et al., 1997). Immunohistochemical staining of human fetal membranes shows that 11β-HSD1 distributes in whole membrane layers (Sun et al., 1997; Wang et al., 2012; Liu et al., 2016a). In the amnion, 11β-HSD1 distributes in both epithelial and fibroblast cells, whereas in the smooth chorion, 11β-HSD1 is localized to trophoblasts, as well as fibroblasts (Sun et al., 1997; Wang et al., 2012; Liu et al., 2016a). Notably, in contrast to the expression of 11β-HSD2 in trophoblasts of human placenta chorionic villi, trophoblasts of the smooth chorion express mainly 11β-HSD1 rather than 11β-HSD2 (Sun et al., 1997). These discrepancies are suggestive of a unique role of 11β-HSD1 rather than a by-product of atrophied villi in the fetal membranes.

Intriguingly, cortisol, despite being a product of 11β-HSD1, induces rather than inhibits 11β-HSD1 expression in both smooth chorion trophoblasts and amnion fibroblasts (Sun et al., 2002; Sun and Myatt, 2003; Li et al., 2006; Yang et al., 2007), thus setting up a positive feedback loop between cortisol

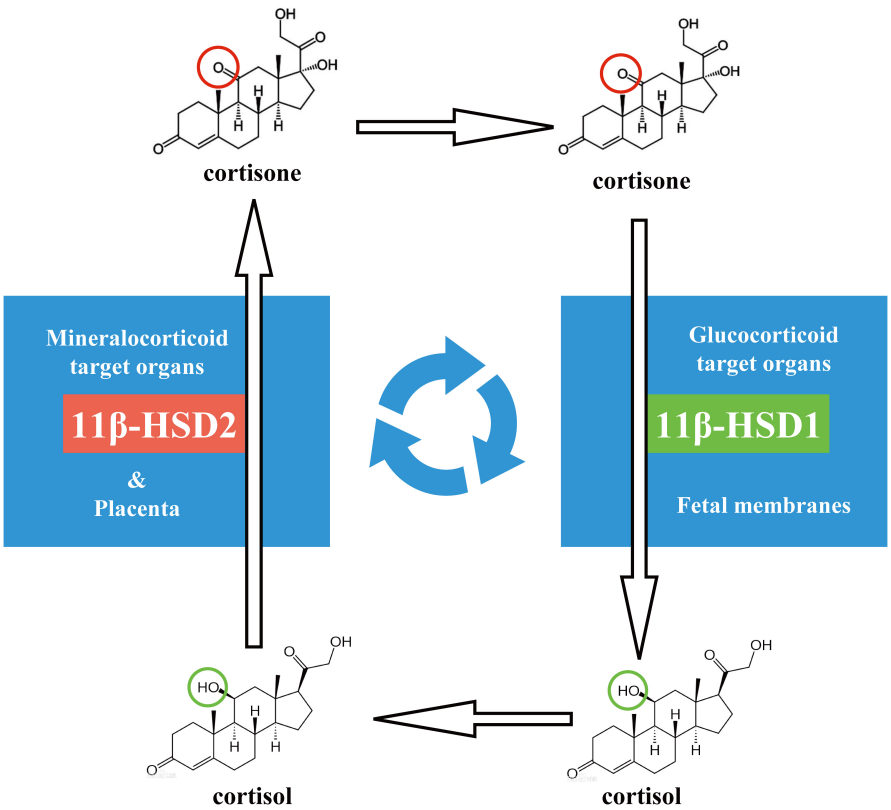

FIGURE 1 | Recycle of cortisol and cortisone between placenta and fetal membranes in human pregnancy.

regeneration and 11β-HSD1 expression in the fetal membranes. This feedforward expression pattern of 11β-HSD1 in the fetal membranes may account for its increasing expression with gestational age (Alfaidy et al., 2003) and its further increase at parturition (Liu et al., 2016a).

The actions of glucocorticoids and proinflammatory cytokines usually oppose each other at sites of inflammation. However, in the fetal membranes, proinflammatory cytokines induce the expression of 11β-HSD1 not only on their own, but also in synergy with glucocorticoids (Sun and Myatt, 2003; Li et al., 2006; Lu et al., 2019c). Given that inflammation is a common cause of both term and preterm birth, and overproduction of proinflammatory cytokines is a common feature of inflammation (Bollapragada et al., 2009; Menon et al., 2010; Romero et al., 2014; Singh et al., 2019), the synergy between glucocorticoids and proinflammatory cytokines in the induction of 11β-HSD1 expression is particularly noteworthy because this synergy is very likely to generate even more cortisol under conditions of chorioamnionitis. These distinct features of 11β-HSD1 expression in the fetal membranes denote again that the expression of 11β-HSD1 in the fetal membranes may be a requisite event in the end of normal gestation and may even be more intriguing in the condition of chorioamnionitis. In non-gestational tissues, proinflammatory cytokines have also been shown to induce 11β-HSD1 expression either on its own (Tomlinson et al., 2001; Yong et al., 2002; Ignatova et al., 2009; Esteves et al., 2014) or in synergy with glucocorticoids (Rae et al., 2004; Kaur et al., 2010), which is regarded as a self-restraining mechanism to avoid over immune responses in inflammation. Is cortisol regeneration by 11β-HSD1 in the fetal membranes simply a self-restraining mechanism to contain inflammation or does it mean more than an anti-inflammatory role in pregnancy? Convincing evidence has accumulated that glucocorticoids derived from fetal adrenal glands can trigger parturition in a number of animal species (Anderson et al., 1975; Flint et al., 1978; First and Bosc, 1979; Morrison et al., 1983; Fowden et al., 2008). However, adrenal glands of human fetus produce mainly dehydroepiandrosterone sulfate (DHEAS) rather than glucocorticoids (Mesiano and Jaffe, 1997; Ishimoto and Jaffe, 2011). This feature of human fetal adrenal glands may also explain why systemic administration of synthetic glucocorticoids such as betamethasone apparently does not induce labor (Craft et al., 1976). This is probably due to the strong negative feedback of synthetic glucocorticoids on the fetal hypothalamic–pituitary–adrenal axis, which may result in diminished production of both DHEAS and cortisol when synthetic glucocorticoids pass through the placenta and enter the fetal circulation. Because DHEAS is a precursor for estrogen synthesis in the placenta, diminished DHEAS will lead to reduced estrogen production (Ogueh et al., 1999), which is essential for preparation of the myometrium for contraction (Mesiano, 2001). Therefore, it is very likely that the feedforward cortisol regeneration by 11β-HSD1 in the

fetal membranes is a compensatory mechanism in parturition for the insufficient cortisol synthesis by fetal adrenal glands in humans. In other words, local regeneration of cortisol in the fetal membranes is probably more important than cortisol derived from fetal adrenal glands in human parturition. This notion is endorsed by increased abundance of glucocorticoid receptor (GR) in the fetal membranes at parturition (Sun et al., 1996), suggesting that cortisol regenerated by 11β-HSD1 in the fetal membranes has enhanced local actions pertinent to human parturition.

ROLE OF CORTISOL REGENERATION IN THE RUPTURE OF FETAL MEMBRANES

During the gestational period, a tough and tensile amniotic sac is required for holding and protecting the fetus bathed in the amniotic fluid. The tensile strength of the fetal membranes is believed to derive mainly from the rich content of collagenous fibers in the compact layer of the amnion (Bourne, 1962; Parry and Strauss, 3rd., 1998; Oyen et al., 2006). Needless to say, the fetal membranes should break for the delivery of fetus at parturition, and rupture of membranes can, in turn, promote labor, suggesting that the fetal membranes are a source of labor initiating signals. As a matter of fact, the fetal membranes are indeed among the gestational tissues that give rise to signals leading to parturition (Parry and Strauss, 3rd., 1998; Menon, 2016; Menon et al., 2016). These multiple source signals may need to work in a coordinating manner to start labor at term. However, intensified signals from the fetal membranes as elicited by membrane rupture or infection can sometimes even start labor alone no matter at term and preterm, which highlights the importance of membrane rupture in parturition. In line with the rupture of membranes, the fetal membranes become increasingly weak toward the end of gestation. Our serial studies indicate that local regeneration of cortisol is an important attributor to membrane weakening and rupture (Liu et al., 2016a; Wang et al., 2016, 2019a; Mi et al., 2017, 2018).

The initial inspiration that has encouraged us to investigate whether cortisol regeneration is involved in membrane rupture comes from the side effects of clinical usage of glucocorticoids. Based on their potent anti-inflammatory properties, synthetic glucocorticoids are widely prescribed to treat a variety of acute and chronic inflammatory conditions (Rhen and Cidlowski, 2005). Among the side effects of glucocorticoids, skin atrophy is frequently encountered (Sterry and Asadullah, 2002; Schoepe et al., 2006). Fibroblasts have been shown to be the target cells of glucocorticoids for this side effect (Nuutinen et al., 2001; Oishi et al., 2002; Schoepe et al., 2006). It has been demonstrated that glucocorticoids reduce tensile strength and elasticity of the skin by decreasing the synthesis of extracellular matrix (ECM) proteins and increasing their turnover in fibroblasts (Cutroneo et al., 1975, 1981; Shull and Cutroneo, 1986; Nuutinen et al., 2001; Oishi et al., 2002; Boudon et al., 2017). For the same reason, glucocorticoids are being used topically to prevent excessive scar formation (Berliner et al., 1967; Jalali and Bayat, 2007). These clinical observations have prompted us to envisage that the feedforward cortisol regeneration in the fetal membranes may be related to ECM remodeling for membrane rupture.

It is now known that the tensile strength of the amnion is largely attributed to collagen I and III contents in the compact layer (Bourne, 1960; Malak et al., 1993; Bryant-Greenwood, 1998). Abundance of collagen I and III in the amnion decreases after the middle trimester to a nadir at term (Skinner et al., 1981; Casey and MacDonald, 1996; Hampson et al., 1997). In preterm premature rupture of membranes, a common cause of preterm labor, collagen content is further decreased (Skinner et al., 1981; Hampson et al., 1997; Stuart et al., 2005). These findings suggest that decreased collagen abundance characterizes the process of membrane rupture. We investigated whether cortisol regenerated in the fetal membranes is involved in the reduction of collagens in cultured primary human amnion fibroblasts, which are the major source of ECM proteins in the amnion compact layer (Casey and MacDonald, 1996). We have demonstrated that cortisol decreases the abundance of collagen I and III protein in a concentration-dependent manner with no effects on their mRNA abundance in amnion fibroblasts. Further mechanistic studies have revealed that cortisol decreases collagen I and III protein abundance by activating the autophagic and proteasome pathways, respectively (Mi et al., 2017, 2018). In addition to collagen I and III, collagen IV is essential for the maintenance of epithelial basal membrane as well as for the ECM structural protein assembling in the amnion (Malak et al., 1993; Bryant-Greenwood, 1998). For this reason, collagen IV is known as another crucial determinant of membrane integrity. By using human amnion fibroblasts, we have found that cortisol also causes the breakdown of collagen IV in a concentration-dependent manner through drastic induction of the expression of matrix metalloprotease 7 (MMP-7), also known as matrilysin, *via* activation of the AP-1 transcription factor (Wang et al., 2019a). These inhibitory effects of cortisol on collagen I, III, and IV have shown to be GR-mediated effects and have been confirmed in amnion tissue explant experiments. These findings are also substantiated by concurrent increases in cortisol, 11β-HSD1, MMP-7, and markers for autophagy and decreases in collagen I, III, and IV in the amnion tissue collected from spontaneous labor with membrane rupture (Liu et al., 2016a; Mi et al., 2017, 2018; Wang et al., 2019a,b).

The tensile strength of the amnion is determined not only by collagen abundance, but also by the degree of their cross-linking. Cross-linked collagens become tough and resistant to the breakdown by MMPs (Vater et al., 1979). It is now known that the cross-linking of collagens is catalyzed by lysyl oxidase (LOX), a copper-dependent amine oxidase (Kagan and Trackman, 1991). It has been shown that LOX protein and enzyme activity decrease dramatically in the amnion with advancing gestational age and further decrease in spontaneous labor with membrane rupture (Casey and MacDonald, 1997; Liu et al., 2016a,b). We have demonstrated that both cortisol and cortisone inhibit LOX expression in human amnion fibroblasts, and the effect of cortisone is abolished with inhibition of 11β-HSD1 (Liu et al., 2016a). Again, these effects have proved to be mediated by GR and have been replicated in amnion tissue explant experiments.

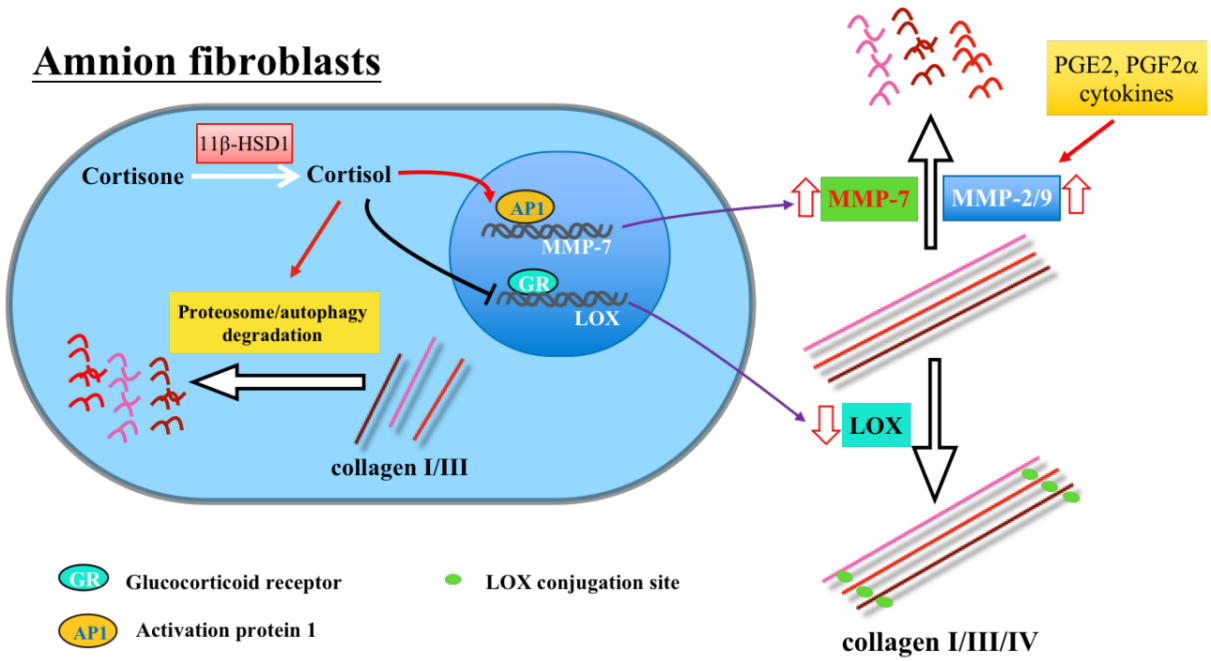

FIGURE 2 | Extracellular matrix (ECM) remodeling effects of cortisol regenerated by 11β-HSD1 in the fetal membranes.

In addition to the effects mediated directly by GR, an alternative mechanism is also present in ECM remodeling effects of cortisol. It has been shown that serum amyloid A1 (SAA1), an acute phase protein produced primarily by the liver, could be produced locally in the fetal membranes (Li et al., 2017), where SAA1 exerts extensive ECM remodeling effects including induction of MMP-1, MMP-2, MMP-8, MMP-9, and MMP-13; inhibition of LOX-like 1; and evoking collagen degradation (Wang et al., 2019b,c). Moreover, SAA1 can induce the expression of 11β-HSD1, and cortisol can in turn stimulate the expression of SAA1 (Lu et al., 2019b,c), thus formulating a mutual reinforcing mechanism in the production of SAA1 and cortisol in the fetal membranes. These findings are suggestive of existence of an alternative mechanism through induction of SAA1 to remodel the ECM structure by cortisol in the fetal membranes. Taken together, all these findings of cortisol's actions on collagens, LOX, and MMP-7 in human amnion fibroblasts are supportive of the view that cortisol regenerated by 11β-HSD1 plays an important role in the ECM remodeling for membrane rupture at parturition (**Figure 2**).

ROLE OF CORTISOL REGENERATION IN THE STIMULATION OF PROSTAGLANDIN E2 AND F2α OUTPUT IN THE FETAL MEMBRANES

Prostaglandins, particularly prostaglandin E2 (PGE2) and prostaglandin F2α (PGF2α), play crucial roles in human parturition (Challis et al., 1997; Romero et al., 2014). They increase myometrium contractility, ripen the cervix, and promote fetal membrane rupture (Challis et al., 1997; Romero et al., 2014). Although all gestational tissues including fetal membranes and maternal decidua/myometrium are virtually capable of PGE2 and PGF2α synthesis, the fetal amnion and maternal decidua/myometrium are recognized to synthesize the most PGE2 and PGF2α, respectively (Duchesne et al., 1978; Mitchell et al., 1978; Okazaki et al., 1981; Rehnstrom et al., 1983). In the amnion, the capacity of PGE2 synthesis in interstitial fibroblasts is approximately 5 times more than the capacity in epithelial cells (Sun et al., 2003). In addition, amnion fibroblasts are also capable of synthesizing PGF2α, although not as much as PGE2 (Guo et al., 2014). As narrated above, glucocorticoids are the most widely used class of anti-inflammatory drugs with prominent inhibitory effects on the synthesis of proinflammatory cytokines, as well as prostaglandins (Rhen and Cidlowski, 2005). These inhibitory effects of glucocorticoids on prostaglandin synthesis are known to be executed mostly through inhibition of cyclooxygenase 2 (COX-2) expression, the rate-limiting enzyme in prostaglandin synthesis (Goppelt-Struebe et al., 1989; Lasa et al., 2001; Lim et al., 2014). However, it has been noted for a long time that there are parallel increases in cortisol and prostaglandin levels in the maternal circulation toward the end of human gestation, and this apparent contradiction is described as a gestational paradox (Casey et al., 1985). Several groups have confirmed that this paradoxical phenomenon holds true in human amnion fibroblasts (Potestio et al., 1988; Zakar et al., 1995; Blumenstein et al., 2000; Sun et al., 2003, 2006). It has been shown that both cortisol and synthetic glucocorticoids stimulate rather than inhibit the expression of COX-2 in human amnion fibroblasts. Moreover, glucocorticoids also induce the expression

of cytosolic phospholipase A2 (cPLA2) in human amnion fibroblasts (Sun et al., 2003; Guo et al., 2008, 2010). Cytosolic phospholipase A2 catalyzes the formation of arachidonic acid, a rate-limiting substrate in prostaglandin synthesis, from membrane phospholipids. The mechanism underlying the paradoxical induction of cPLA2 and COX-2 by glucocorticoids is fascinating because glucocorticoids inhibit the expression of proinflammatory cytokines in amnion fibroblasts at the same time. We set out to delineate this paradoxical mechanism and it turned out to be a very complicated mechanism. It is revealed that glucocorticoids induce cPLA2 and COX-2 expression through stimulation of the cAMP/PKA pathway with subsequent activation of multiple transcription factors including CREB and STAT3, and so on (Zhu et al., 2009; Guo et al., 2010; Wang et al., 2015; Lu et al., 2017, 2019a). Interestingly, the classical inflammatory transcription factor, nuclear factor κB, is nevertheless inhibited by glucocorticoids (Guo, 2010), which is responsible for the inhibition of proinflammatory cytokine expression in human amnion fibroblasts, a situation resembling most of non-gestational tissues.

As depicted above, the amnion is also capable of synthesizing PGF2α, although not as much as PGE2 (Guo et al., 2014). There are multiple pathways for PGF2α synthesis in amnion fibroblast. In addition to PGF synthase (PGFS)–catalyzed formation of PGF2α from PGH2, PGF2α can also be converted from PGE2 by the enzyme carbonyl reductase 1 (CBR1) (Ziboh et al., 1977). We have found that cortisol significantly induces the synthesis of PGF2α through induction of CBR1 but not PGFS in amnion fibroblasts (Sun et al., 2003; Guo et al., 2014). This induction of CBR1 by cortisol was revealed to be GR-mediated enhancement of CBR1 transcription (Guo et al., 2014).

During the gestational period, there is abundant expression of prostaglandin degrading enzyme 15-hydroxyprostaglandin dehydrogenase (PGDH) in trophoblasts of the smooth chorion, which is known as a prostaglandin barrier (Cheung et al., 1992; Johnson et al., 2004). PGDH catalyzes NAD^+-linked oxidation of 15 (S)-hydroxyl group of prostaglandins resulting in inactivation of their biological activities (Tai et al., 2002). Therefore, PGE2 and PGF2α synthesized in the amnion are mostly blocked from reaching the uterus by this barrier. Studies have shown that the abundant expression of PGDH in chorion trophoblasts is maintained mostly by progesterone (Challis et al., 1999; Patel and Challis, 2002). However, this maintenance is eventually undermined by increasing concentrations of cortisol derived from the feedforward regeneration through 11β-HSD1 (Patel et al., 1999a,b; Patel and Challis, 2002). The involvement of 11β-HSD1 is supported by findings that inhibition of PGDH by cortisone is reversed by 11β-HSD1 inhibitor (Patel et al., 1999b). It has been suggested that accumulating cortisol may compete with progesterone for progesterone receptor, thereby attenuating the maintaining effect of progesterone on PGDH expression and leading to progressively undermined prostaglandin barrier in the smooth chorion at term (Patel et al., 2003). Like the situation of cPLA2 and COX-2 induction by glucocorticoids, the inhibition of PGDH by glucocorticoids in the smooth chorion is also a kind of paradox, which is in marked contrast to the induction of PGDH expression by glucocorticoids in most of non-gestational tissues (Xun et al., 1991; Tong and Tai, 2005). All these findings of glucocorticoids on the induction of cPLA2/COX-2/CBR1 in amnion fibroblasts and inhibition of PGDH in chorion trophoblasts are supportive of a role of cortisol regeneration in the stimulation of PGE2 and PGF2α output either by induction of their synthesis or by inhibition of their degradation in the fetal membranes (**Figure 3**).

SUMMARY AND PERSPECTIVES

In pregnancy, there is increasing cortisol regeneration by 11β-HSD1 toward the end of gestation. 11β-HSD1 is expressed in virtually all cell types in the fetal membranes. Although the smooth chorion of the fetal membranes is considered as atrophied chorionic villi, the fetal membranes including the smooth chorion are endocrinologically active in pregnancy. Cortisol regeneration by 11β-HSD1 is one of such endocrine activities. Cortisol regenerated by 11β-HSD1 is involved not only in ECM remodeling for membrane rupture but also in the upregulation of PGE2 and PGF2α outputs. Both are requisite events for the onset of parturition. In addition to the actions described in this review, it remains to be uncovered whether cortisol regenerated by 11β-HSD1 in the fetal membranes possesses other unknown actions pertinent to the onset of labor. Another interesting issue remains to be clarified is the apparent contradictions between the concurrent induction of prostaglandins and inhibition of proinflammatory cytokines by glucocorticoids in the fetal membranes. Because

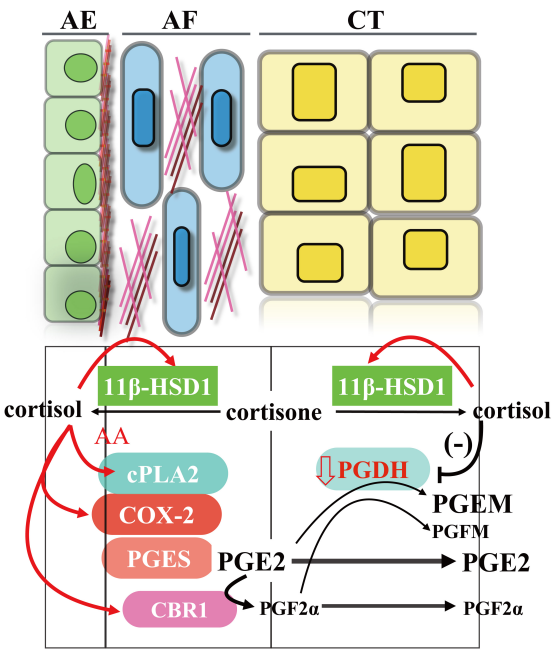

FIGURE 3 | Induction of PGE2 and PGF2α output by cortisol regenerated through 11β-HSD1 in human fetal membranes. AE, amnion epithelial cells; AF, amnion fibroblasts; CT, smooth chorion trophoblasts; AA, arachidonic acid.

both prostaglandins and proinflammatory cytokines are prolabor factors involved in ECM remodeling and uterine contractile activities (Keelan et al., 2003), it would be interesting to understand how these actions of cortisol on prostaglandins and proinflammatory cytokines are balanced in the fetal membrane at parturition, particularly in the situation of chorioamnionitis. Nevertheless, these effects of cortisol on ECM remodeling and prostaglandin output might be even enhanced in chorioamnionitis, given the potentiation of cortisol regeneration by proinflammatory cytokines. These apparent contradictions may represent a unique feature how glucocorticoids work locally in the fetal membranes in the promotion of labor. Simultaneous inhibition of proinflammatory cytokines and stimulation of prostaglandins by glucocorticoids may avoid deleterious effects of proinflammatory cytokines on the fetus on the one hand but save the labor promoting effects of prostaglandins on the other hand. Finally, it would be helpful to find a suitable animal model to replicate those findings in humans, such as the expression pattern of 11β-HSD1 across gestational age and those actions of glucocorticoids on ECM remodeling, prostaglandin synthesis, and degradation in the fetal membranes. What is more important is to test whether local artificial manipulation of 11β-HSD1 and GR expression in the fetal membranes can indeed change the course of gestation in the right animal model. Taking all together, we can conclude that cortisol regeneration in the fetal membranes is not a coincidental but a requisite event in parturition.

AUTHOR CONTRIBUTIONS

KS conceived the idea. KS, W-SW, and C-MG contributed to manuscript writing and figure preparation.

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Premature Rupture of Membranes and Severe Weather Systems

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There has long been anecdotal evidence of early labor and delivery in severe weather events leading to preterm birth. In particular, significant barometric pressure changes are associated with hurricanes and bomb cyclones. Some authors have related these low pressure weather events to premature rupture of fetal membranes, hypothesizing that the membranes act as an inflated balloon and respond directly to pressure changes. In this article, the key literature including data supporting this hypothesis is reviewed. A simple numerical model, based on a competition between the driving and resisting forces for fetal membrane rupture, is presented. This model provides a quantitative mechanism for membrane failure in the context of storms with low atmospheric pressure. Other sequelae of severe storms that are unrelated to fetal membrane rupture are also discussed. Labor and delivery in the context of major weather events should be understood in a holistic framework that includes both exogenous and endogenous factors relevant to the pregnant patient.

Keywords: preterm, premature, PROM, chorioamnion, fetal, membranes, rupture, failure

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1. INTRODUCTION

There have been many articles written in the popular press about a potential association between major weather events, such as hurricanes, and early childbirth (LaFrance, 2016; Blau, 2017; Bolluyt, 2018). There is some evidence in the scientific literature in support of such an association, particularly between low levels of barometric pressure and premature rupture of the fetal membranes (PROM) (Polansky et al., 1985; Akutagawa et al., 2007). In this context, "premature" refers not to the gestational age of the pregnancy (instead that is called "preterm") but the rupture of the membranes prior to the onset of labor. Other studies have found no association between barometric pressure and PROM (Marks et al., 1983). However, the methodology associated with many articles in this genre has been criticized for being insufficient in terms of patient numbers, weather-related data, inadequate control populations, or other deficiencies.

The current work aims to examine this hypothesis of preterm PROM associated with significant drops in barometric pressure associated with major weather events. A number of key studies in this area will be reviewed in the first section of this paper. The physical mechanics of PROM and barometric pressure change will be elucidated with an analytical model in the following section. This will be followed by discussion and conclusions, emphasizing the need for further study on the relationship between major weather events and late-gestation pregnant women, with a view toward potential intervention via evacuation or watchful and conservative medical management.

2. KEY LITERATURE

An early study completed at the University of Iowa Hospital and published in 1985 (Polansky et al., 1985) showed that PROM occurred more often when the barometric pressure decreased 3 h beforehand. The results of this study further showed that the onset of labor for matched control patients within the same geographical area were not associated with barometric pressure changes. If there was an increase in barometric pressure instead of a drop in pressure, PROM was not affected. This study noted that previous authors examining the issue of PROM and barometric pressure suffered methodological deficiencies in their studies, and that Polansky et al. had designed their study to avoid these flaws. The article postulated that barometric pressure could create a gradient across the chorioamniotic membranes to maintain in utero pressure, but suggested that prostaglandins or other biochemical mechanisms could also be responsible.

A 1997 study focussed on the onset of labor associated with significant decreases in barometric pressure (King et al., 1997). Although not focussed on PROM or membrane rupture, a significant increase in the onset of labor was found in the 24 h after a significant barometric pressure drop and not in the 24 h prior. This study in a journal aimed at nurse-midwives, recommended that low pressure weather systems should be monitored in the context of labor and delivery units and that this association should be mentioned to pregnant women in childbirth classes.

A landmark retrospective study covering 1997–2003 and published in 2007 (Akutagawa et al., 2007) demonstrated that deliveries increased on days with a larger change in barometric pressure in a statistically significant manner. Rupture of the membranes, including premature rupture, was associated with lower barometric pressures, in this study defined by a cut-off value of 758.1 mm Hg (1010.7 hPa in their manuscript). The authors note that labor pains bare associated with both hormones and the autonomic system, and that these both could be affected by local weather and by more general environmental changes. However, they clearly postulate that the membrane rupture and low barometric pressure are not just associated but causal, consistent with the model developed in the following section.

An extensive series of studies of the physical strength of the chorioamnion membrane was performed by Oyen et al. (2004), Oyen et al. (2006), Calvin and Oyen (2007), and Chua and Oyen (2009) showing the decrease in membrane strength with gestational age. These studies focussed on labored versus C-section deliveries and the effects of twin pregnancies, emphasizing endogenous effects. In addition, these authors developed a mechanics-based framework for prediction of preterm birth as a function of changes in pregnancy status, such as polyhydramnios (increased amniotic fluid pressure and volume) or infection within the chorioamnion membrane (Oyen et al., 2004). Here, that mechanics model is applied to consider exogenous factors, in particular the barometric pressure change associated with severe weather events, to test the hypothesis that hurricanes could cause PROM.

3. MODEL AND ANALYSIS

A simple model is constructed here for rupture of the fetal (chorioamnion) membrane as a function of substantial decreases in atmospheric pressure. Considerations of fetal membrane rupture will follow that of reference Oyen et al. (2004) where a similar model was examined for endogenous effects such as chorioamnionitis or polyhydramnios. The basis of the model is a competition between stress in and strength of the membrane. In this context, stress is a driving force for mechanical rupture of the membrane, deriving from exogenous causes. In contrast, strength is a resisting force for mechanical failure of the membrane, deriving from endogenous effects. Mechanical failure or rupture of the membrane occurs when the strength of the membrane is exceeded by the applied stress. It is assumed that membrane strength is a function of gestational age alone, and that there is no influence of weather systems on the material properties resisting rupture. In contrast, it is assumed here that the mechanical stress in the membrane is a function of barometric pressure and no consideration of endogenous physiological factors such as decreased membrane strength from infection or changes in other physiological processes is driving mechanical failure. It is entirely likely that the mechanical changes modeled here in isolation are combined with additional contributions to increase the likelihood of PROM in the difficult circumstances of a severe storm. We consider first the membrane strength values based on experimental measurements of puncture force.

Failure data for membranes were taken from the raw data from the study of puncture force $F_{\max}$ as a function of gestational age (GA, Oyen et al., 2006). The data were split into two GA groups, $GA \leq 29$ and $GA \geq 30$ weeks. The raw data were fit by linear regression for each GA range (**Figure 1**). These regression lines were used as the input force $F_{\max}$ as a function of gestational age for calculations of membrane strength, using the equation

$$\sigma_{\mathrm{f}} = \frac{1}{h}\left(\frac{F_{\max} E h}{6\pi R}\right)^{1/2} \tag{1}$$

where h is the membrane thickness, E is the membrane elastic modulus, and $R = 1.6$ mm was the probe radius used in the puncture studies (Oyen et al., 2004, 2006). Here h was taken as 250 μm and E was taken as 5 MPa based on published data (Helmig et al., 1993). The failure strength σ_{f} shows an increase with GA up to 30 weeks and a decrease thereafter (solid line, **Figure 2**). We consider next the membrane stress and its changes with barometric pressure.

The membrane stress as a function of fluid pressure was computed according to Laplace's law, as utilized for this context in (Oyen et al., 2004):

$$\sigma = p\frac{r}{2h} \tag{2}$$

where r is the radius of the sac bounded by the membrane and h is again the membrane thickness. The same estimates were used as previously (Oyen et al., 2004) for the size of the sac, linearly

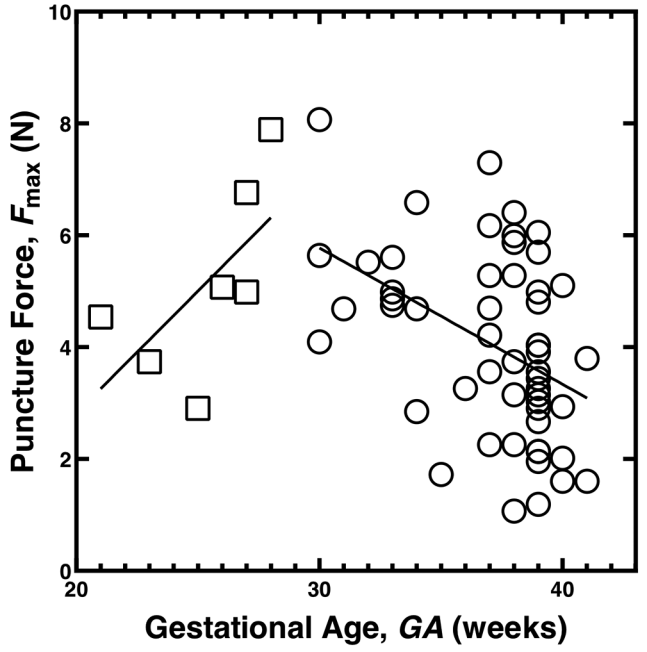

FIGURE 1 | Puncture force for the chorioamnion membrane as a function of gestational age, data from the studies (Oyen et al., 2004, 2006). The data have been split into two groups and fit with linear trend lines, $GA \leq 29$ weeks (open squares) and $GA \geq 30$ weeks (open circles).

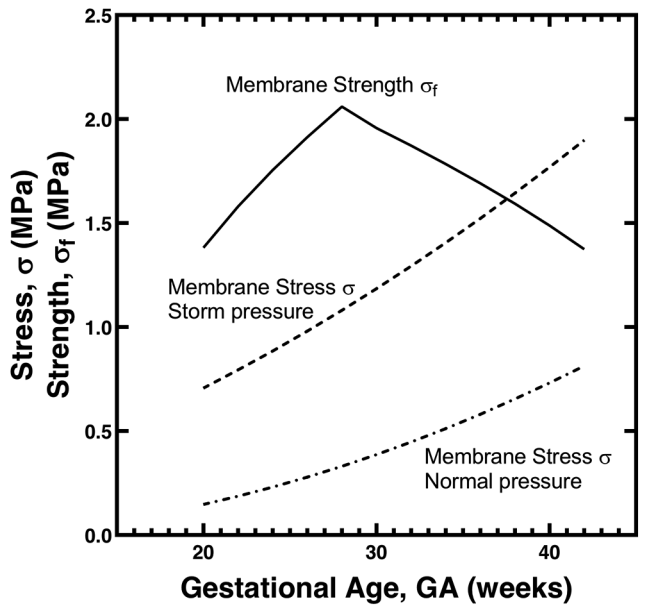

FIGURE 2 | Model for CA membrane stress σ and strength σ_f as a function of gestational age *GA*. The strength values are calculated from the data in **Figure 1** using Equation (1); the stress values for normal and storm atmospheric pressures are calculated from Equation (2) with parameter values as described in the text.

increasing from 7 cm at 20 weeks to 13 cm at 40 weeks GA. Amniotic fluid pressure was taken from Weiner et al. (1989) where the data were digitized and the best fit line was taken as $p = 0.661 * GA - 5.278$. This gives a fluid (gage) pressure of approximately 20 mm Hg above ambient (Faber et al., 1992) at full gestation of GA = 40 weeks (**Figure 2**, lower dashed line). This baseline was assumed to be for a normal atmospheric pressure of 760 mm Hg. For storm conditions, the atmospheric pressure was assumed to decrease to 730 mm Hg, and thus 30 mm Hg was added to each amniotic fluid pressure due to the change in computing the gage pressure (**Figure 2**, upper dashed line). It is critical to note that there are no free parameters in this modeling approach, nor are there any pre-factors that are adjusted without mechanism or associated literature data. The calculations of stress and strength are independent save for the shared parameter of membrane thickness (h).

The membrane stress is on the order of the membrane strength in the case of normal atmospheric pressure. The value of chorioamnion strength of about 1 MPa near term is consistent with a large review study that found a similar value across a large number of studies and measurement methods (Chua and Oyen, 2009). The stress and strength curves will be shifted slightly up or down depending on the choice of parameter values (p, E, r, h) used in the model and on the experimentally-measured force values used (F_{max}). However, the general trend is clear, that a 30 mm Hg drop in atmospheric pressure associated with a storm has the potential to raise the membrane stress significantly, and increase the likelihood that the membrane stress exceeds the membrane strength for a range of late-term gestational ages. Thus, a causal mechanism of membrane rupture due to pressure changes is numerically plausible.

4. DISCUSSION

A series of key studies over the course of the last 35 years have demonstrated that there is a clear association of birth with substantial drops in barometric pressure (King et al., 1997), and that this is in particular related to premature rupture of the fetal membranes (Polansky et al., 1985; Akutagawa et al., 2007). A simple model (Oyen et al., 2004) is adapted to consider this association, treating the membranes as a bubble and considering the trade-off between decreasing membrane strength as full term approaches, and increasing membrane stress associated with barometric pressure drops. It is found that a 30 mm Hg pressure drop, consistent with major hurricanes or bomb cyclones (which can occur in winter and not be tropical) is sufficient to provide a mechanistic association between membrane rupture and low atmospheric pressure (**Figure 2**).

The assumption in the model that atmospheric pressure could influence the stress in the chorioamnion does rely on the membrane being exposed to a pressure difference between the amniotic fluid and the surrounding ambient conditions. It seems more likely that this could be in the case in relatively late gestation when the cervical mucous plug has started to discharge or is missing entirely. This is particularly true if the cervix has also started to dilate, in which case the membranes begin to be directly exposed to ambient air via the vaginal canal.

The model here was plotted (**Figure 2**) for a pressure drop from a typical atmospheric pressure of 760 mm Hg to a representative storm pressure of 730 mm Hg. This pressure drop of 30 mm Hg in the model was associated with a prediction of membrane rupture at GA = 37.5 weeks at a stress value of 1.62

MPa. A pressure drop of 25 mm Hg, with all other parameters remaining unchanged, predicts rupture at GA = 39 weeks at a stress of 1.54 MPa, a drop of 35 mm Hg predicts rupture at GA = 36.1 weeks at a stress of 1.68 MPa, and a drop of 40 mm Hg predicts rupture at GA = 34.7 weeks at a stress of 1.75 MPa. Thus, a more severe storm in terms of decrease from baseline atmospheric pressure is associated with predictions of membrane rupture at younger gestational ages but greater stresses, since the membrane strength is assumed to decrease with GA (Oyen et al., 2006). These model predictions as noted assume all other model parameters are unchanged, and thus serve as a baseline indicator of the severity of storm pressure changing in isolation of all other potentially changing factors.

Membrane rupture due to low atmospheric pressure is not the only potential mechanism associated with early labor and delivery relative to weather events. Large increases in atmospheric pressure in a relatively short time have also been associated with premature delivery (Akutagawa et al., 2007) although the mechanism for that must be different than the one described herein, perhaps associated with other physiological changes in blood pressure or volume. Less extreme meteorological factors have been associated with both preterm delivery and PROM, including sharp changes in temperature and humidity and strong winds (Yackerson et al., 2008). Such weather conditions were also found to be associated with obstetrical complications such as placental abruption and pre-eclampsia (Yackerson et al., 2007).

There are many possible reasons as to why hurricanes can affect pregnancy, including complications in labor and delivery and subsequent morbidity in the neonate. For example, exposure to hurricanes during a pregnancy can increase the chance of a newborn having to rely on a ventilator (Currie and Rossin-Slater, 2013). The same authors found higher chances of an infant having aspirated meconium, a sign of fetal distress, and that hurricanes were generally associated with low birth weight. There was evidence in this study of significant sequelae in infants even when exposure to storms was early in the pregnancy, as in the first or second trimester (Currie and Rossin-Slater, 2013). Different potential explanations are discussed in this thorough work, including the effect of maternal stress, evacuation out of the path of the storm, and impact of the storm on delivery of medical services in the community. All of these results take on increased importance given our increasing awareness that abnormal conditions surrounding the newborn's arrival can cause poor outcomes for the child not just in the immediate aftermath of birth but also later in life.

One further issue thus far not addressed at all in the literature linking barometric pressure changes and pregnancy is race and ethnicity, and the corresponding health disparities that arise in preterm birth (Culhane and Goldenberg, 2011). The March of Dimes in the US rates states on maternal and infant health and in particular focuses on preterm birth (2019). Many of the states with the worst grades on the March of Dimes scale (2019) coincide with states that are also the most likely to be directly hit in a hurricane (Griggs, 2017). The lack of information linking these two topics presents an interesting opportunity for future research by an interdisciplinary team considering aspects from socioeconomics to biomechanics.

5. CONCLUSION

Labor and delivery in the context of major weather events should be understood in a holistic framework that includes both exogenous and endogenous factors. Management of pregnancies in the path of large storms with significantly low barometric pressures could include education of both health care practitioners and of expectant mothers about this phenomenon. It is entirely likely that evasive action such as evacuation could be warranted for some late-stage pregnancies directly in the path of significant barometric pressure drops on the basis of both the literature and on the basis of the numerical model presented herein. This study motivates further research on this subject, in the context of gathering far more detailed experimental data from regions with high risk for serious weather incidents to better inform expectant mothers and their health care providers. Such research will gain relevance as the occurrence of severe weather events increases due to climate change.

DATA AVAILABILITY STATEMENT

The dataset analyzed for this study can be found at Oyen (2020) and is related to that in the previous publication (Oyen et al., 2006).

AUTHOR CONTRIBUTIONS

MW provided background research in the form of an annotated bibliography and participated in all aspects of writing and editing the manuscript. MO performed the analysis and created the figures in discussion with MW, and participated in all aspects of writing and editing the manuscript.

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PERSPECTIVE
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Toxicant Disruption of Immune Defenses: Potential Implications for Fetal Membranes and Pregnancy

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In addition to providing a physical compartment for gestation, the fetal membranes (FM) are an active immunological barrier that provides defense against pathogenic microorganisms that ascend the gravid reproductive tract. Pathogenic infection of the gestational tissues (FM and placenta) is a leading known cause of preterm birth (PTB). Some environmental toxicants decrease the capacity for organisms to mount an immune defense against pathogens. For example, the immunosuppressive effects of the widespread environmental contaminant trichloroethylene (TCE) are documented for lung infection with *Streptococcus zooepidemicus*. Group B *Streptococcus* (GBS; *Streptococcus agalactiae*) is a bacterial pathogen that is frequently found in the female reproductive tract and can colonize the FM in pregnant women. Work in our laboratory has demonstrated that a bioactive TCE metabolite, S-(1, 2-dichlorovinyl)-L-cysteine (DCVC), potently inhibits innate immune responses to GBS in human FM in culture. Despite these provocative findings, little is known about how DCVC and other toxicants modify the risk for pathogenic infection of FM. Infection of the gestational tissues (FM and placenta) is a leading known cause of PTB, therefore toxicant compromise of FM ability to fight off infectious microorganisms could significantly contribute to PTB risk. This Perspective provides the current status of understanding of toxicant-pathogen interactions in FM, highlighting knowledge gaps, challenges, and opportunities for research that can advance protections for maternal and fetal health.

Keywords: fetal membranes, toxicant pathogen interactions, preterm birth (PTB), pregnancy, trichloroethylene (TCE)

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INTRODUCTION

Preterm birth (PTB), or birth <37 weeks gestation, is a significant health problem with lasting consequences. Preterm birth affects more than 1 in 10 babies in the United States as well as globally (March of Dimes et al., 2012; Martin et al., 2019). Babies born preterm are at increased risk for numerous adverse health outcomes later in life, including neurological (Allin et al., 2006), lung (Pike and Lucas, 2015), and intestinal issues (Behrman et al., 2007). In a recent study, Grosse et al. (2017) estimated total medical costs associated with PTB in the United States to be between $6 and 14 billion per year. The fetal membranes (FM), which surround and protect the fetus during pregnancy, play a critical role in both term and preterm labor. In addition to providing a physical barrier, the FM are an

important line of defense against pathogenic microorganisms that ascend the reproductive tract (Romero et al., 2007). Notably, pathogenic infection of the gestational tissues (FM and placenta) is a leading known cause of PTB (Goldenberg et al., 2000, 2008).

Epidemiology studies have identified a diverse array of factors associated with PTB (Goldenberg et al., 2008; Ferguson et al., 2013, 2019; Torchin and Ancel, 2016; Vogel et al., 2018). These include exposure to a range of toxic substances including air pollution (Liu et al., 2019), cigarette smoke (Soneji and Beltran-Sanchez, 2019), polyfluoroalkyl substances (Sagiv et al., 2018), polybrominated diphenyl ethers (PBDEs; Peltier et al., 2015), phthalate esters (Ferguson et al., 2014), lead (Taylor et al., 2015), and arsenic (Ahmad et al., 2001). Additional factors include infection with pathogenic bacteria (Bianchi-Jassir et al., 2017) and exposure to high outdoor air temperatures (Zhong et al., 2018; Gronlund et al., 2020). On a mechanistic basis, these exposures are thought to act by triggering oxidative stress and/or inflammatory pathways that are part of the normal labor process of weakening the membranes (Menon et al., 2011; Romero et al., 2014; Wallace et al., 2016; Ha et al., 2018). While biologically plausible, these mechanisms remain poorly understood.

Despite known examples of toxicant-induced immunosuppression occurring in organs such as the lung (Aranyi et al., 1986; Mitchell et al., 2009; Selgrade and Gilmour, 2010), toxicant mediation of immune responses to bacterial infection in FM is largely unexplored. This review focuses on our current understanding about environmental toxicants, pathogenic bacteria and interactions between the two in FM. Due to the potential lifelong health impacts of PTB (Allin et al., 2006; Behrman et al., 2007; Pike and Lucas, 2015) and the critical role that the membranes play in healthy pregnancy (Menon and Moore, 2020), a deeper understanding of these interactions has significant public health implications.

ANATOMY AND FUNCTION OF THE FETAL MEMBRANES

The FM are a heterogenous tissue with multiple cell types that make up two distinct layers, the inner amnion (surrounding the fetus) and the outer chorion (Strauss, 2013). The amnion layer is composed of a single amnion epithelial cell layer and dense layer of collagen fibrils synthesized by fibroblasts (Verbruggen et al., 2017). The chorion is composed of trophoblasts that are in close contact with maternally derived decidual cells (Wang et al., 2018; Menon and Moore, 2020). The FM also include a small number of resident innate immune cells (macrophages and monocytes) (Osman et al., 2003).

The culmination of a healthy pregnancy is marked with increased prostaglandin secretion, activation of matrix metalloproteinases, and recruitment of immune cells, leading to myometrial contractions, rupture of the membranes, and cervical ripening, respectively (Vadillo-Ortega et al., 1996; Hernandez-Guerrero et al., 2000; Challis et al., 2009; Yellon, 2019). Although our understanding of the role of FM in the initiation of labor remains incomplete, it is widely accepted that they contribute to the parturition pathway. As pregnancy progresses, the FM secrete increasingly more cytokines and chemokines, which leads to prostaglandin synthesis and release (Mesiano, 2007; Kota et al., 2013) as well as immune cell recruitment in the gestational compartment (Osman et al., 2003). Because the FM abut the uterine muscle (myometrium), they are important as a source of prostaglandins that stimulate uterine contractions in labor.

Furthermore, in normal pregnancies, FM undergo a process of weakening leading up to rupture soon after the start of uterine contractions (Menon, 2016; Menon et al., 2016). Molecular signaling pathways, such as oxidative stress and inflammation, as well as mechanical forces contribute to the weakening of the membranes near term, a process characterized by cellular senescence and aging of the membranes (Menon et al., 2016). Rupture usually occurs in a structurally weak region of the membranes with a thinner chorion that overlies the cervix, referred to as the zone of altered morphology (ZAM; McLaren et al., 1999; McParland et al., 2003; Marcellin et al., 2017).

Premature rupture of the FM, or PROM, is characterized by rupture of the FM more than one hour before the onset of labor. PROM occurring after 37 weeks of pregnancy typically presents relatively few complications. However, pPROM, or preterm premature rupture of the FM (i.e., PROM that occurs prior to 37 weeks of gestation) is associated with severe adverse pregnancy outcomes and is frequently associated with asymptomatic intrauterine infection (Mercer, 2004; Caughey et al., 2008; Huang et al., 2018). Examples of associated adverse neonatal outcomes include respiratory distress syndrome, pulmonary hypoplasia (Nourse and Steer, 1997; Linehan et al., 2016), and neurological outcomes (Manuck and Varner, 2014). pPROM affects around 1–3% of pregnancies (Huang et al., 2018).

FETAL MEMBRANES AS A TARGET OF BACTERIAL PATHOGENS

Intrauterine bacterial infection is well established as a cause of PTB (Romero et al., 2014). It is estimated that intrauterine infection accounts for at least 25–40% of PTBs (Goldenberg et al., 2008). Both placenta and FM from preterm and pPROM pregnancies have been shown to be more likely to contain bacterial DNA and a higher level of diversity in bacterial species compared to term pregnancies (Jones et al., 2009). Pathogenic bacteria associated with pPROM and PTB include species from genera such as *Staphylococcus*, *Escherichia, Mycoplasma, Ureaplasma*, and *Streptococcus* (Larsen and Hwang, 2010; Oh et al., 2010; Fortner et al., 2014; Zeng et al., 2014; Kong et al., 2019).

The predominant mechanism by which bacteria enter the gestational compartment causing intrauterine infection is through the ascending pathway by which bacteria first colonize the vagina and cervix, migrate to and then cross the FM, and then colonize the amniotic cavity and fetus (Goldenberg et al., 2000). Therefore, the FM play a critical role as a barrier to bacterial entry.

In addition to providing a physical barrier to protect against infection, the FM provide crucial immunological defense against pathogenic microorganisms that ascend the reproductive tract. The FM actively secrete antimicrobial peptides, such as

human beta defensins, lactoferrin, and cathelicidin, to inhibit bacterial infection (Kjaergaard et al., 1999; King et al., 2007a,b; Boldenow et al., 2013). Furthermore, the choriodecidual cells as well as resident innate immune cells are capable of secreting proinflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α, which help signal for additional immune cell recruitment (Challis et al., 2009; Yockey and Iwasaki, 2018). Proinflammatory cytokines can also potentially trigger increased release of prostaglandins and proteases, which are key molecular triggers of parturition (Norwitz et al., 1992; Mitchell et al., 1993; Brown et al., 1998; Young et al., 2002; Myatt and Sun, 2010; Romero et al., 2014). Even when bacteria do not infect the amniotic compartment, these proinflammatory responses to bacterial infection in the FM can lead to adverse pregnancy and neonatal outcomes (Adams Waldorf et al., 2011; Burd et al., 2012; Garcia-Flores et al., 2018).

Much of what is currently known about toxicant-bacteria interactions in FM comes from experiments using either *Streptococcus agalactiae*, commonly known as Group B *Streptococcus* (GBS). Group B *Streptococcus* infection in pregnant women is the leading cause of infectious neonatal morbidity and mortality in the United States (Verani et al., 2010). Group B *Streptococcus* induces preterm labor in non-human primates (Gravett et al., 1996; Boldenow et al., 2016). In women, GBS infection is associated with PTB at less than 32 weeks gestation (Hillier et al., 1991) and with chorioamnionitis, an inflammation of the chorion layer of the FM (Anderson et al., 2007). A recent publication from our laboratory showed that GBS inoculation caused a release of molecular effectors of parturition (matrix metalloproteinases and prostaglandin E2) from human FM explant punches *in vitro* (Park et al., 2018). In addition, pathway analysis of transcriptomic responses showed that pathways related to inflammation and PTB were activated by GBS inoculation (Park et al., 2018). Studies from our laboratory showed that a metabolite of trichloroethylene (TCE), a common environmental contaminant, modifies innate immune response to GBS in FM explants (Boldenow et al., 2015). Other groups have shown similar effects with other toxicant-bacteria combinations (e.g., carbon monoxide and *Escherichia coli* (Klimova et al., 2013). Although rarely explored, interactive effects between pathogens and toxicants in gestational tissues are plausible and have significant implications for maternal and fetal health.

FETAL MEMBRANES AS A TARGET OF ENVIRONMENTAL TOXICANTS

Pregnant women are exposed to a multitude of diverse environmental contaminants through drinking water, food packaging, air pollution workplace exposures, and other sources (Mitro et al., 2015). Ubiquitous environmental contaminants such as lead, cadmium, PBDEs, bisphenol A, and phthalates have been detected in human FM (Miller et al., 2009; Kot et al., 2019) and amniotic fluid (Miller et al., 2012; Geer et al., 2015), demonstrating that contaminants can come into contact with the FM either through blood flow to the decidua or via the amniotic fluid. Numerous epidemiology studies have found associations between exposures to environmental toxicants and increased risk of pPROM. These include toxic substances such as lead (Huang et al., 2018), ambient air pollution (Wang et al., 2019) and cigarette smoke (England et al., 2013). These epidemiology studies along with the detection of toxicants in human FM support the role of FM as a target of toxicant effects related to adverse pregnancy outcomes.

Toxicants Activate Pathways Involved in Fetal Membrane Rupture and PTB

Consideration of the FM as a mediator of toxicant effects is plausible based on their important role in membrane rupture and in the initiation of labor. As recently reviewed by Menon (2016), Menon et al. (2019), and Menon and Moore (2020), the FM contribute to the activation of labor and membrane rupture through a variety of molecular signaling pathways involving hormones, inflammatory cytokines, phosphorylated MAPK p38, reactive oxygen species and prostaglandins. Pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-8 are secreted by the FM and promote the production of prostaglandins and proteases in the gestational compartment (Norwitz et al., 1992; Mitchell et al., 1993; Brown et al., 1998; Young et al., 2002; Myatt and Sun, 2010). Prostaglandins play a direct role in stimulating uterine contractions and cervical ripening, and proteases and ROS contribute to the weakening of the FM (Woods, 2001; Romero et al., 2014). The p38 MAPK pathway is critical for the initiation of cellular senescence and FM weakening, ultimately leading to membrane rupture (Menon et al., 2014). Increased generation of ROS in the gestational compartment is thought to activate the p38 pathway, leading to membrane senescence, damage to collagen, and weakening of the membranes in preparation for rupture in both term labor and pPROM (Woods, 2001).

Toxicants such as cigarette smoke extract and PBDEs activate one or more of these pathways in *in vitro* models of FM tissue or cells. For example, PBDEs induced oxidative stress, p38 MAPK activation and increased expression of cyclooxygenase-2 (a rate limiting enzyme of prostaglandin production) in human amnion epithelial cells (Behnia et al., 2015). Similarly, Menon et al. (2014) showed that cigarette smoke extract induced oxidative stress (assessed via formation of 3-nitrotyrosine staining) and activated the p38 MAPK pathway in FM explants *in vitro*. In addition, the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), often referred to as dioxin, increased expression of protease genes in human amnion epithelial cells (Abe et al., 2006) and increased a marker of senescence (β-galactosidase) in a FM "organ-on-chip" system consisting of primary human amnion epithelial cells co-cultured with decidual cells (Richardson et al., 2019). Thus, toxicology studies support molecular mechanisms that may explain epidemiological associations between toxicant exposures and adverse pregnancy outcomes mediated by the FM. However, several aspects of these phenomena, such as the thresholds of exposure and potential dimorphic responses based on fetal sex, remain largely unexplored.

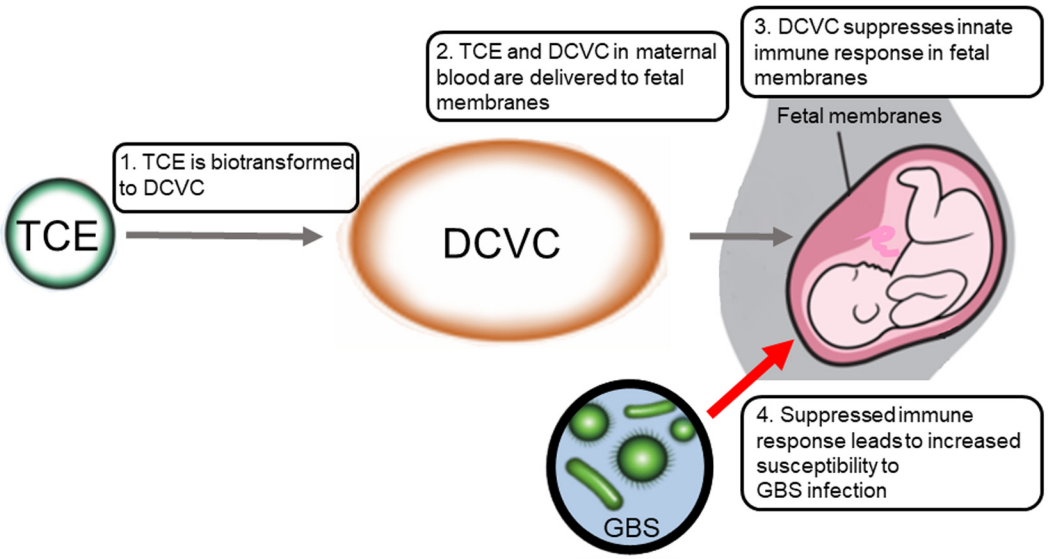

FIGURE 1 | Proposed model of TCE immunosuppression in fetal membranes leading to increased susceptibility to GBS infection during pregnancy.

TOXICANT-PATHOGEN INTERACTIONS: IMPACT ON FETAL MEMBRANES

Whereas several mechanisms have been identified which present plausible explanations for FM toxicity, immunomodulation in conjunction with bacterial infection remains an important but understudied phenomenon in gestational tissues. In a 2010 review, Feingold et al. (2010) highlighted the need for environmental toxicology research to incorporate interactions with infectious pathogens such as bacteria and viruses. Feingold et al. (2010) described four potential toxicant-pathogen interactions that could lead to disease: (1) toxicant and pathogen are both needed to cause disease; (2) pathogen and toxicant are individually capable of causing disease; (3) the chemical toxicant modifies the pathogen which leads to disease; and (4) the pathogen modifies the toxicant which leads to disease. In the same journal issue, Birnbaum and Jung (2010) called for increased attention to environmental health and infectious disease, noting that they can act concurrently, antagonistically, or synergistically. Despite this call to action, little research on toxicant-pathogen interactions during pregnancy has been conducted in the last decade.

Toxicant-Pathogen Co-treatment Leads to Enhanced Inflammation

Some toxicants have been shown to enhance pathogen-stimulated oxidative stress pathways and pro-inflammatory responses in gestational tissues. For example, some PBDEs increased *E. coli*-stimulated IL-1β and IL-6 secretion and COX-2 expression, as well as reduced *E. coli*-stimulated IL-10 release in human placental explants (Peltier et al., 2012; Arita et al., 2018c). Similarly, TCDD increased bacteria-stimulated PGE_2 and COX-2 gene expression and decreased IL-10 secretion (Peltier et al., 2013). Notably, the PBDE and TCDD effects were observed in the absence of impacts on explant viability and in placenta tissue obtained from both term (Arita et al., 2018c) and preterm (Peltier et al., 2012, 2013) stages of pregnancy, suggesting that immunomodulatory effects can occur throughout gestation. In addition, tributyltin enhanced *E. coli*-stimulated IL-6 release from placental explants (Arita et al., 2018a). Another study found that the flame retardant chemical tetrabromobisphenol A (TBBPA) increased the *E. coli*-induced release of IL-6 and TNF-α (Arita et al., 2018b). Research continues to be limited on how these toxicants modify bacterial host response in the FM and *in vivo*. Given the important nature of the FM in pPROM and PTB it is imperative that more research be conducted on toxicant-pathogen interactions in the FM.

Immunosupression as a Mechanism of Toxicity

Whereas some toxicants enhance inflammation and immune responses, others have demonstrated immunosuppressive effects (Selgrade, 2007). Examples of toxic substances that suppress immune responses include alcohol, cigarette smoke, and air pollution, all of which have been shown to inhibit macrophage phagocytosis (Karavitis and Kovacs, 2011). Epidemiology studies have found associations between decreased antibody responses to vaccinations in children exposed to perfluorinated compounds (Grandjean et al., 2012) and polychlorinated biphenyls (Heilmann et al., 2006). Immunosuppressive effects of toxicants have also been observed in gestational tissues. For example, TBBPA and tributyltin both inhibited bacteria-stimulated IL-1β secretion in placental explants (Arita et al., 2018a,b).

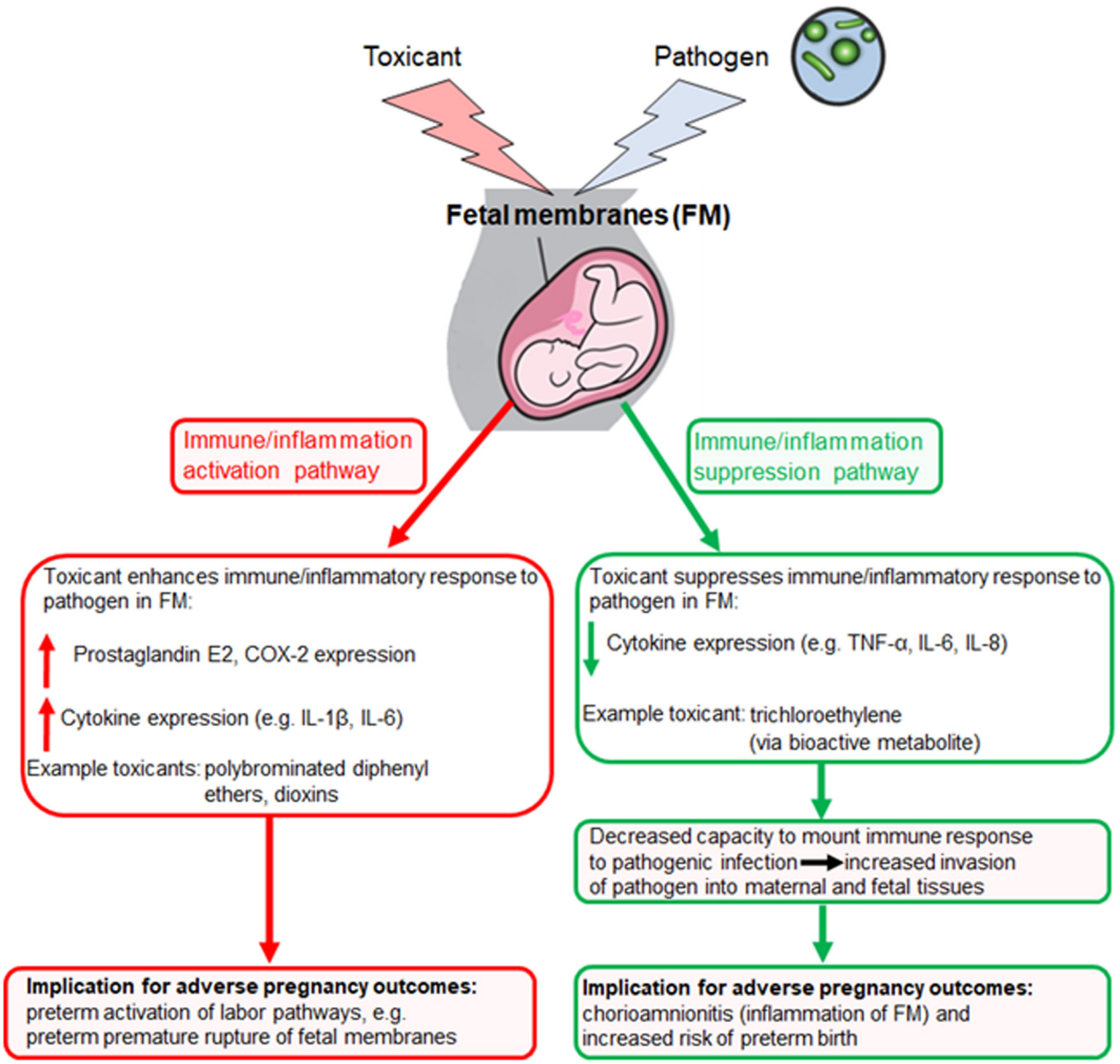

FIGURE 2 | Proposed pathways for toxicant activation or suppression of inflammation/immune responses in the fetal membranes with potential implications for pregnancy outcomes. Multiple environmental toxicants have been identified that either enhance or suppress immune responses in the fetal membranes, particularly in models of pathogenic infection. Both mechanisms of toxicity have potentially significant implications for adverse pregnancy outcomes, e.g., early activation of labor pathways (activation) or decreased capacity for membrane tissue to mount a defense against pathogens (suppression). These pathways may not be mutually exclusive.

Immunomodulatory Effects of Trichloroethylene

The common environmental contaminant TCE is a well-documented example of a compound with immunosuppressive effects. Trichloroethylene is a chlorinated volatile organic solvent commonly used as an industrial metal degreaser (Waters et al., 1977; Chiu et al., 2013). Trichloroethylene is ranked #16 on the U.S. Agency for Toxic Substances and Disease Registry's Priority List of Hazardous Substances and is a common environmental contaminant found in approximately 800 Environmental Protection Agency-designated Superfund sites (Wong, 2004; Chiu et al., 2013). Trichloroethylene is classified as a "known human carcinogen" (Guha et al., 2012) and is a renal and hepatic toxicant However, effects of TCE on gestational tissues have been minimally explored. Because of its continued industrial use and widespread persistent environmental contamination, TCE exposure continues to pose a threat to human health through ingestion of contaminated drinking water and inhalation of the volatilized chemical (Watson et al., 2006; Dumas et al., 2018). Trichloroethylene and

its metabolites are detected in the blood of pregnant women exposed via inhalation and transfer across the placenta has been indicated by detection in the umbilical vein and artery (Beppu, 1968; Laham, 1970). Trichloroethylene and its metabolites are also found in the placenta and amniotic fluid of exposed pregnant mice (Ghantous et al., 1986). Thus, the effect of TCE and its downstream metabolites on gestational tissues in exposed women is of relevant concern.

The immunomodulatory effects of TCE are well documented in rodent and epidemiology studies. Mice co-treated with TCE and *Streptococcus zooepidemicus* showed increased mortality, decreased bacterial clearance from the lungs, and decreased alveolar phagocytosis (Aranyi et al., 1986; Selgrade and Gilmour, 2010). Trichloroethylene also suppressed activity of natural killer cells isolated from exposed rats (Wright et al., 1991). Immunosuppressive effects are observed in humans exposed to TCE. For example, lymphocyte counts as well as circulating levels of proinflammatory cytokines IL-6 and TNF-α were lower in exposed workers compared to controls (Hosgood et al., 2011; Xueqin et al., 2018). In a 2009 review, Cooper et al. (2009) concluded that "studies in mice and humans support an etiologic role of TCE in autoimmune disease." It appears that metabolism is required for at least some of TCE's immunotoxicity, because inhibition of the TCE metabolizing enzyme CYP2E1 mitigates some of these effects (Griffin et al., 2000). Despite these compelling findings, few researchers have investigated this phenomenon in gestational tissues such as the FM. Because the FM play a vital role in protecting the fetus and gestational compartment from pathogenic infection during pregnancy, an increased understanding of how environmental contaminant exposures modify FM responses to infection could greatly improve our ability to identify populations at risk for bacterial infection and associated adverse pregnancy outcomes.

TCE Metabolite Suppression of Immune Responses to Bacteria in Fetal Membranes

Work in our laboratory demonstrated that the bioactive TCE metabolite S-(1,2-dichlorovinyl)-L-cysteine (DCVC) inhibits innate immune responses to GBS. These findings were observed in FM tissue explants (tissue cultures established from FM obtained from planned caesarian deliveries). Explants co-treated with GBS and DCVC showed decreased expression of TNF-α, IL-1β, and IL-8 compared to those treated with GBS alone (Boldenow et al., 2015). Two other TCE metabolites (TCA and DCA) showed no effect (Boldenow et al., 2015). Importantly, the concentrations of DCVC used (5–10 μM) were within the range of metabolite blood concentrations in female volunteers exposed to airborne TCE at the current occupational exposure limit (Lash et al., 1999; Agency for Toxic Substances and Disease Registry, 2007). Moreover, the immunomodulatory effects of DCVC occurred in the absence of any effect on overall GBS viability. The cytokine suppression occurred not only in response to GBS, but also in response to lipoteichoic acid and lipopolysaccharide (virulence factors expressed by multiple species of bacteria) (Alexander and Rietschel, 2001; Ginsburg, 2002), suggesting that the observed effects were not pathogen specific.

Suppression of cytokine expression has important implications for innate immune responses in FM. Cytokines play important roles during bacterial infection, such as the recruitment of immune cells. Thus, suppression of these cytokine responses could lead to decreased recruitment of immune cells during bacterial infection, leading to prolonged or more severe pathogenic infections during pregnancy. Prolonged or more severe infections could in turn lead to pPROM, PTB or other adverse pregnancy outcomes such as neonatal sepsis.

Figure 1 summarizes the major events in the proposed mechanism by which TCE exposure could lead to increased susceptibility to GBS infection. Few epidemiology studies have assessed associations between TCE exposure and PTB. Studies thus far have found associations with small for gestational age, low birth weight and birth defects but not PTB (Bove et al., 2002; Forand et al., 2012; Ruckart et al., 2014). However, these studies did not report on presence or absence of maternal pathogenic infection as a variable and obtaining accurate assessments of TCE exposure is challenging (Bove et al., 2002). Future epidemiology studies focusing on potential toxicant-pathogen interactions could greatly improve our understanding of whether phenomena observed in FM models *in vitro* translate to *in vivo* human outcomes.

DISCUSSION

Despite intriguing findings, numerous aspects of toxicant-pathogen interactions in FM need to be clarified in order to reach conclusions about the implications for maternal or fetal health outcomes. Although DCVC suppression of innate immune responses could exacerbate GBS infection, proinflammatory pathways are also critical in the activation of parturition, meaning that DCVC could also suppress activation of labor processes (**Figure 1**). A better understanding of mechanisms underlying these phenomena would clarify the true level of risk for adverse pregnancy outcomes due to GBS infection combined with TCE exposure. In addition, findings thus far have only been observed in FM tissue *in vitro*. While useful, these models lack a number of tissue interactions between the decidua and the chorionic layer of the FM as well as maternal immune responses to infection. Validating these findings in pregnant animal models co-treated with GBS and TCE could provide important clarification in this area. Furthermore, clarification is needed on whether DCVC is the sole metabolite of TCE responsible for immunosuppressive effects or if downstream metabolites play a role. Improved understanding of virulence factors that allow bacteria such as GBS to evade the defenses of the FM and colonize the amniotic fluid and/or fetus would also represent a significant step forward. Fetal sex and gestational age are other potentially important variables that were not considered in prior studies of the FM. Finally, TCE is far from the only toxicant known to have immunosuppressive effects. For example, perfluorinated chemicals such as perfluorooctanoic acid have recently generated concern due

to observed immunosuppressive effects (Shane et al., 2020) and therefore should be investigated for interactions with pathogens in the context of pregnancy. Other classes of chemicals that have demonstrated immunosuppressive effects include aromatic hydrocarbons, benzene and metals such as lead and arsenic (National Academy of Sciences, 1992).

Some studies have noted both immunosuppressive and immune activation effects for the same toxicant. For example, Arita, et al. observed an increase in E. coli-induced TNF-α in placental explants treated with TBBPA, whereas IL-1β secretion was reduced (Arita et al., 2018b). This is not surprising given the inherent complexity of immunological signaling pathways. For a given toxicant, it is possible that both immune activation and suppression could occur to differing degrees simultaneously or in sequence, which is especially important to recognize when utilizing *in vitro* models. For example, if cultured FM are exposed to the toxicant and pathogen simultaneously then the toxicant may not diffuse into the tissue before the pathogen stimulates the early TNF-α response, but the toxicant could still inhibit the later IL-1β response. Additionally, the toxicant may act to inhibit or activate different molecular pathways within the immune system. For example, the toxicant could be inhibiting caspase, which is needed for IL-1β secretion, while simultaneously activating TNF-α (Thornberry et al., 1992). If immune activation predominates, adverse pregnancy events may include preterm activation of labor pathways which could lead to premature rupture of the FM. If immune suppression is the dominant process, adverse events could include increased pathogenic infiltration into the gestational compartment due to inadequate FM immune response. The mechanisms determining whether suppression or activation predominate in the FM in response to toxicants are not currently well understood but are likely mediated by a number of factors including the dose of toxicant, stage of pregnancy, strain or species of pathogen or duration of toxicant exposure (e.g., chronic vs. acute exposure). For example, naturally occurring immunological changes occurring throughout pregnancy include a progressive increase in the number and responsiveness of circulating neutrophils (Aghaeepour et al., 2017). Therefore, being exposed to a toxicant and/or pathogen late in pregnancy may favor immune activation whereas a different response may be observed with exposure earlier in pregnancy. Whether toxicant immune activation, suppression or a more complex interaction between the two, is the most relevant to the FM for a given toxicant-pathogen interaction is difficult to predict, further highlighting the need for additional research on this topic.

In summary, limited studies have shown that toxicants can potentially modify immune responses in the FM through both "immune/inflammation activation" and "immune/inflammation suppression" pathways (see **Figure 2**). Because current research into these phenomena has relied mostly on *in vitro* models of gestational cells and tissues, further research is needed to determine whether effects observed *in vitro* are replicated in FM *in vivo*. *In vitro* models are necessarily removed from the inherent complexity of the *in vivo* immune system. Studies using animal models would improve our understanding of how toxicants affect immune responses in the FM in an intact organism. Further research could improve our understanding of toxicant-pathogen interactions during pregnancy and potentially identify populations at risk for adverse pregnancy outcomes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

EB, SH, and RL-C proposed the original idea for the manuscript. All authors wrote and edited the manuscript and have seen and approved the final version of the submitted manuscript.

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Healing Mechanism of Ruptured Fetal Membrane

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Preterm premature rupture of membranes (pPROM) typically leads to spontaneous preterm birth within several days. In a few rare cases, however, amniotic fluid leakage ceases, amniotic fluid volume is restored, and pregnancy continues until term. Amnion, the collagen-rich layer that forms the load-bearing structure of the fetal membrane, has regenerative capacity and has been used clinically to aid in the healing of various wounds including burns, diabetic ulcers, and corneal injuries. In the healing process of ruptured fetal membranes, amnion epithelial cells seem to play a major role with assistance from innate immunity. In a mouse model of sterile pPROM, macrophages are recruited to the injured site. Well-organized and localized inflammatory responses cause epithelial mesenchymal transition of amnion epithelial cells which accelerates cell migration and healing of the amnion. Research on amnion regeneration is expected to provide insight into potential treatment strategies for pPROM.

Keywords: premature rupture of membrane, fetal membrane, amnion, macrophage, wound healing

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IS pPROM IRREVERSIBLE?

Preterm premature rupture of membranes (pPROM) is a leading cause of preterm birth (Menon and Richardson, 2017). Fetal membrane rupture has traditionally been regarded as an irreversible process: the mean latency period from membrane rupture to delivery is 12 days at 20–26 weeks of gestation and 4 days at 32–34 weeks of gestation (Parry and Strauss, 1998). In some cases, however, ruptured fetal membranes can spontaneously "reseal": Johnson reported that membrane resealing, defined as cessation of fluid leakage and negative nitrazine test, occurred in 24 cases of 208 pPROM patients (11.5%) in all 5,937 deliveries (Johnson et al., 1990). In addition, we know that the membrane repairs itself and heals spontaneously after amniocentesis (Borgida et al., 2000). These findings suggest that, although most women who experience pPROM deliver spontaneously within several days, the amnion has the capacity for wound healing *in vivo*.

CAUSES OF pPROM

About 30% of pPROM cases are caused by intra-amniotic infection, whereas the other 70% are unrelated to infection (Romero et al., 1988). pPROM cases that are unrelated to infection are caused by smoking, low body mass index, maternal stress or undernutrition, oxidative stresses, intrauterine bleeding, and iatrogenic factors such as amniocentesis or fetoscopy.

Romero et al. reported that intra-amniotic inflammation occurs in 37% of cases of preterm labor before 37 weeks of gestation. Interestingly, the rate of inflammation with infection was only 11%, whereas that of sterile inflammation in the absence of bacteria was 26% (Romero et al., 2014). They suggested that sterile intra-amniotic inflammation might be caused by damage-associated molecular patterns (DAMPs), such as high-mobility group box1 (HMGB1), and concluded that sterile inflammation is a more common contributor to preterm labor than bacterial infection.

DAMPs are believed to play a major role in the pathophysiology of sterile inflammation. Specifically, when a tissue is damaged, intracellular components and molecules such as HMGB1, nucleic acids, heat-shock proteins, adenosine triphosphate, hydrogen peroxide, and calcium ions are released (Kono and Rock, 2008). Uric acid and S100 proteins are associated with pPROM (Friel et al., 2007; Nadeau-Vallee et al., 2016). These DAMPs are recognized by toll-like receptors and receptor for advanced glycation end products (RAGE), leading to activation of inflammatory pathways such as NF-κB and AP-1, which yield sterile inflammation (Akira et al., 2006; Xia et al., 2017). Although DAMPs are released when tissue is damaged, they are also signals of tissue repair. Whereas pPROM initiated by bacterial infection requires immediate delivery to avoid fetal infection, the numerous pPROM cases that are unrelated to infection may be eligible for expectant management.

HEALING OF FETAL TISSUES: THE ROLES OF MACROPHAGES

The healing mechanisms of adult tissue are divided into four overlapping stages: (1) hemostasis, (2) inflammation, (3) migration and proliferation, and (4) resolution and remodeling (Sonnemann and Bement, 2011). In contrast with adult tissues, the healing of fetal tissue is much simpler (Sonnemann and Bement, 2011): inflammation is suppressed to a minimum, fetal tissue is usually not vascularized, and granulation tissue is usually not formed. These characteristics of fetal wound healing enable the tissue to heal quickly and scarlessly (Cordeiro and Jacinto, 2013). For example, when fetal skin is injured, actin and myosin proteins aggregate in the injured epidermis to form acto-myosin complexes that cause contraction of the tissue and shrinkage of the area of injury. These cellular structures stimulate migration of the epidermis and closure of the wound.

Remarkably, macrophages are recruited to injury sites to facilitate healing of fetal tissues. Circulating monocytes migrate to injury sites where they differentiate into tissue macrophages, and tissue-resident macrophages are also involved in wound healing (Jenkins et al., 2011).

Macrophages are roughly divided into two types (Murray and Wynn, 2011), classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages) (Gordon and Martinez, 2010). Wound healing is facilitated by M2 macrophages (Murray and Wynn, 2011). These cells release growth factors, such as transforming growth factor (TGF-β) and platelet-derived growth factors (PDGF), which activate damaged epidermis and fibroblasts. TGF-β plays a major role in the differentiation of fibroblasts from myofibroblasts. These cells migrate and contract, as well as release tissue inhibitor of metalloproteinases (TIMPs), which inhibits matrix metalloproteinases (MMPs) and prevents over-destruction of tissues. Myofibroblasts also release collagen and repair damaged sites in conjunction with macrophages, which also release MMPs and TIMPs and remodel wounded tissue. Subsequently, macrophages phagocytose debris and damaged extracellular matrix (ECM) to clean the wounded tissues.

HEALING OF AMNION IN ORGAN CULTURE

In an experiment reported by Devlieger et al. (2000b), small holes were generated with a biopsy punch in the centers of human fetal membrane sample. Interestingly, increased cellularity, survival, and proliferation were limited at the tissue border and the rupture did not heal even after 12 days. This result suggests that amnion cannot heal by itself; rather, the help of other cells such as immune cells are necessary for wound healing in the amnion.

ANIMAL MODELS OF FETAL MEMBRANE HEALING

Amnion has a high tensile strength; in fact, the strength of the fetal membrane is provided exclusively by the amnion (Parry and Strauss, 1998). Although fetal membrane structures differ among mammals, humans, and several experimental animals including mice, rats, rabbits, and sheep all have similar amnion structure; they also all have amnion in the most superficial layer of the fetal membrane (Carter, 2016). Thus, animal models are useful for the study of ruptured human fetal membranes *in vivo*.

The first histological observations of the healing process in fetal membranes were conducted in rats. Pioneering work by Sopher (Sopher, 1972) demonstrated that puncturing rat gestational sacs with a 21-gauge needle on day 15 of gestation resulted in a proliferation of amnion mesenchymal cells at the edge of the amnion within 24 h. Further, she showed that the thickened edge of the amnion was covered by epithelial cells and confirmed that wound closure occurred within a few days. Similarly, in a rabbit model, amnion integrity recovered to 40% of its initial value within 30 days of puncture (Deprest et al., 1999). The healing process of rabbit pPROM involves matrix remodeling by MMPs and TIMPs (Devlieger et al., 2000a).

Using a mouse model, we investigated the mechanisms of wound healing of fetal membranes. On day 15 of pregnancy, fetal membranes were mechanically ruptured with sterile needles of various sizes through the myometrium. Ruptured fetal membranes were clearly observed after 6 h and healing began within 24 h. Our mouse study revealed that the closure of such ruptures was complete within 48–72 h (Mogami et al., 2017). Consistent with Sopher's study, we observed an aggregation of amnion mesenchymal cells at the edge of the amnion at 24 h.

Interestingly, this thickened edge was covered by a monolayer of epithelial cells. The proinflammatory cytokines IL-1β and TNF were quickly increased at the fetal membrane rupture site. When a 26-gauge needle was used to create a small rupture, this increase in proinflammatory cytokines returned to basal levels around 24 h. When a 20-gauge needle was used to create a larger rupture, the puncture-induced increases in these cytokines persisted for a longer time. At the same time, IL-10, an anti-inflammatory cytokine, increased at the ruptured site, decelerating inflammation. IL-10 assists in wound healing, as shown by the finding that overexpression of IL-10 in mice accelerates skin healing (Peranteau et al., 2008). In contrast, chronic inflammation conditions such as diabetic ulcers delay wound healing, suggesting the importance of a balance between inflammation and anti-inflammation for complete and organized wound healing. In the amnion, well-controlled switching from a pro- to an anti-inflammatory state seems to be necessary for repair.

We observed an aggregation of macrophages around the sterile ruptured amnion (Mogami et al., 2017). These macrophages were fetal-derived and were probably recruited from the amniotic fluid, although they may have been amnion-resident macrophages. These fetal-derived macrophages released IL-1β and TNF at the ruptured site. In contrast with the typical wound healing process in adults, migration of neutrophils was rarely observed. Perhaps this is not surprising given the absence of infection and the sterile nature of the inflammatory stimulus. Yet, this raises questions regarding the role of these inflammatory cytokines at the ruptured amnion. We tested the function of these cytokines through *in vitro* scratch assays using primary human amnion cells. IL-1β and TNF caused significant acceleration of amnion epithelial cell migration. They did not, however, alter amnion mesenchymal cell migration. Importantly, the shape of the amnion epithelial cells changed, assuming a more spindle-like configuration (similar to that of mesenchymal cells) at the edge of migration. These spindle-shaped cells were immunoreactive for vimentin, suggesting that these wounded epithelial cells were undergoing epithelial-mesenchymal transition (EMT). *In vivo*, similarly, vimentin-positive cells can be observed scattered in the epithelial layer of the ruptured amnion in mice, suggesting that EMT occurs *in vivo* as well. EMT is known to speed up cell migration, which in turn speeds up wound closure. Our results imply that EMT provides more mesenchymal cells to the wounded amnion, where these cells then synthesize and release extracellular matrices such as collagen to strengthen the injured site. Richardson and Menon also reported that EMT occurs during amnion healing (Richardson and Menon, 2018) and that mesenchymal-epithelial transition (MET) occurs with the help of IL-8 once amnion closure is complete. In addition, Richardson et al. also recently showed that oxidative stresses activate the p38 MAPK pathway, which causes EMT in the fetal membrane (Richardson et al., 2020). Taken together, these results suggest that EMT is a key mechanism involved in stimulating amnion healing in the presence of sterile inflammation.

There is a concern that the healing properties of the amnion differ among species. In rabbits, for example, relatively small punctures created with a 14-gauge needle spontaneously healed to 41.7% of their initial state (Deprest et al., 1999), whereas relatively large ruptures created with a 1 cm hysterotomy did not heal at all (Papadopulos et al., 1998). Similarly, in a mouse model, the amnion healed at a slower rate after being punctured with a 20-gauge needle than after being punctured with a 26-gauge needle (Mogami et al., 2017). We speculate that the reported variation in healing potential depends on the initial size of the rupture rather than on species differences.

IMPORTANCE OF "SCAFFOLDS" FOR HEALING TISSUES

ECM scaffolds have recently received attention as a fascinating mechanism involved in wound healing acceleration and tissue regeneration (Eming et al., 2014). For example, a type-1 collagen patch preserved contractility and protected cardiac tissue from injury in a mouse myocardial infarction model, accompanied by attenuated left ventricular remodeling, diminished fibrosis, and formation of a network of blood vessels within the infarct (Serpooshan et al., 2013; Wei et al., 2015). Porcine urinary bladder ECM scaffold implantation improved the regeneration of muscle in volumetric muscle loss in rodents as well as in five human patients; perivascular stem cell mobilization was seen in connection with this procedure (Sicari et al., 2014). Bioengineered biomaterials have been clinically applied to replace and restore the skin, heart valves, trachea, and tendons (Lutolf and Hubbell, 2005; Berthiaume et al., 2011).

The application of biomaterials to ruptured membranes has been attempted in such animal models as rabbits, sheep, and rats (Zisch and Zimmermann, 2008). When gelatin sponge plugs were used in ewes and rhesus monkeys, for example, rupture sites were found to be intact at term (Luks et al., 1999).

Previously, we showed that application of a collagen matrix assisted amnion healing in a mouse model of sterile pPROM (Mogami et al., 2018). In this model, a type I collagen gel was injected into mechanically-ruptured sites on murine fetal membranes immediately after puncture. The collagen gel was immediately solidified due to the animal's body temperature such that it formed a collagen matrix layer beneath the ruptured amnion (**Figure 1A**). Interestingly, macrophages were trapped in this layer of collagen (**Figure 1B**). Moreover, this injection of collagen thickened the healing site, presumably stimulating more collagen synthesis by the mesenchymal cells in the amnion. We found vimentin-positive mesenchymal cells in the wounded layer of the amnion, suggesting that EMT occurs in this situation, as we had previously reported in our mouse pPROM model. Collagen injection dramatically increased the overall healing rate to 90%, whereas an injection of phosphate buffered saline alone resulted in a healing rate of only 40%. We concluded that scaffold formation at the wounded site in the amnion stimulates wound healing through at least two mechanisms. First, the scaffold provides a base for migrating amnion cells to cover the wound. Second, the matrix scaffold traps, concentrates, and localizes wound healing macrophages.

Application of collagen to the rupture site has also been tested in a rabbit pPROM model. In that study, amnion integrity was diminished by the injection of a collagen "plug" compared

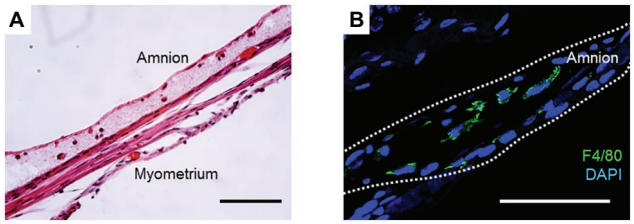

FIGURE 1 | (A) H&E staining of collagen-injected fetal membrane at ruptured site at 72 h. Note that a collagen gel layer was formed beneath the amnion, and immune cells were trapped inside the gel.
(B) Immunofluorescence staining for F4/80 (green) and DAPI (blue) in the collagen layer at 48 h. Bars, 50 μm. All animals were handled and euthanized in accordance with the standards of humane animal care described by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, using protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Southwestern Medical Center.

to myometrial closure alone. This result is different from ours. We speculate that this is because we injected a collagen "gel" in liquid form to the rupture site using a syringe, such that the gel spreads immediately after injection around the rupture site rather than forming a "plug" as in the rabbit study (Papadopulos et al., 1998). The formation of a plug might block the migration of amnion cells. Our collagen gel, in contrast, formed a collagen layer beneath the amnion in our mouse model. This layer serves as a scaffold for migrating amnion cells and traps macrophages. Thus, it never interferes with the healing process. The form of biomaterials (liquid or solid) and the means of their application (injection or patch) may thus be as important as the material type itself.

The effectiveness of biomaterial scaffolds has been observed in other tissues. Bone and cardiac muscle-derived tissue ECM scaffolds for traumatic muscle wounds in mice improved tissue regeneration (Sadtler et al., 2016). In this study, macrophages and immune cells were increased at the injured site, allowing these immune cells to be polarized into a type 2 immune state. Therefore, providing a scaffold is a good strategy for stimulating healing of ruptured amnion. The least invasive means of accomplishing this *in vivo* remains under active investigation.

CONCLUSION

Based on several previous studies, we speculate that the amnion might be capable of healing. Several cell types coordinate and orchestrate wound healing in the fetal membranes, including amnion epithelial cells that differentiate into mesenchymal cells, migrating mesenchymal cells, differentiating resident macrophages, and recruited fetal macrophages. ECM scaffolds could support spontaneous healing of the amnion not only by promoting the migration of amnion cells but also by polarizing macrophages into a type-2 phenotype. The mechanisms by which the amnion heals itself represent a new field of study in which a great deal more research must be done to clarify how this healing process works.

AUTHOR CONTRIBUTIONS

HM and RW wrote the manuscript.

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frontiers in Physiology

REVIEW
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The Role of Danger Associated Molecular Patterns in Human Fetal Membrane Weakening

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The idea that cellular stress (including that precipitated by stretch), plays a significant role in the mechanisms initiating parturition, has gained considerable traction over the last decade. One key consequence of this cellular stress is the increased production of Danger Associated Molecular Patterns (DAMPs). This diverse family of molecules are known to initiate inflammation through their interaction with Pattern Recognition Receptors (PRRs) including, Toll-like receptors (TLRs). TLRs are the key innate immune system surveillance receptors that detect Pathogen Associated Molecular Patterns (PAMPs) during bacterial and viral infection. This is also seen during Chorioamnionitis. The activation of TLR commonly results in the activation of the pro-inflammatory transcription factor Nuclear Factor Kappa-B (NF-kB) and the downstream production of pro-inflammatory cytokines. It is thought that in the human fetal membranes both DAMPs and PAMPs are able, perhaps via their interaction with PRRs and the induction of their downstream inflammatory cascades, to lead to both tissue remodeling and weakening. Due to the high incidence of infection-driven Pre-Term Birth (PTB), including those that have preterm Premature Rupture of the Membranes (pPROM), the role of TLR in fetal membranes with Chorioamnionitis has been the subject of considerable study. Most of the work in this field has focused on the effect of PAMPs on whole pieces of fetal membrane and the resultant inflammatory cascade. This is important to understand, in order to develop novel prevention, detection, and therapeutic approaches, which aim to reduce the high number of mothers suffering from infection driven PTB, including those with pPROM. Studying the role of sterile inflammation driven by these endogenous ligands (DAMPs) activating PRRs system in the mesenchymal and epithelial cells in the amnion is important. These cells are key for the maintenance of the integrity and strength of the human fetal membranes. This review aims to (1) summarize the knowledge to date pertinent to the role of DAMPs and PRRs in fetal membrane weakening and (2) discuss the clinical potential brought by a better understanding of these pathways by pathway manipulation strategies.

Keywords: amnion, danger associated molecular pattern, Pattern Recognition Receptor, fetal membrane, Toll-like receptor, preterm premature rupture of fetal membrane, pathogen associated molecular pattern

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UNDERSTANDING FETAL MEMBRANE RUPTURE IS IMPORTANT TO IMPROVE THE HIGH RATE OF PRETERM BIRTH

The human fetal membranes are an often-overlooked tissue by those studying the mechanisms of parturition. They are disregarded, as many consider that term fetal membranes are a dead tissue, or simply a membranous extension of the placenta. However, many researchers have successfully highlighted it's importance by culturing tissue explants (Zaga et al., 2004; Astern et al., 2012) and isolated cells (Kendal-Wright et al., 2010; Sato et al., 2016), revealing its role as a complex conduit between the mother and fetus (Hadley et al., 2018). It has a large surface area for signaling and clearly contributes to the inflammation that is an established signature of parturition (Romero et al., 2007), regardless of whether it is precipitated by infection (Gomez-Lopez et al., 2018).

Parturition involves several distinct, yet integrated, physiological events; cervical ripening and dilation, contractility of the myometrium, rupture of the membranes, placental separation and uterine involution (Christiaens et al., 2008). All of these processes need to occur in a coordinated manner for the successful delivery of the fetus at term. Thus, desynchrony or the dysregulation of these events can lead to Preterm Birth (PTB) via a number of different pathways (Goldenberg et al., 2008). Approximately 20% of all preterm deliveries are by Cesarean section for maternal or fetal indications (Christiaens et al., 2008). Of the remaining cases, around a third are caused by premature preterm rupture of the membranes (pPROM), 20–25% result from intra-amniotic infection, and the remainder due to premature uterine contractions (Christiaens et al., 2008). However, approximately 60% of all preterm deliveries still remain unexplained (Christiaens et al., 2008). Epidemiological studies have suggested that preterm delivery is a condition that clusters in families (Strauss et al., 2018), and that the incidence of pPROM and the other causes of PTB differ among ethnic groups (Manuck, 2017). Although about 50% of all PTB is due to infection, antibiotics that successfully treat the infection do not halt PTB (Gravett et al., 2007). Once the fetal membranes rupture, they are beyond rescue as there is no commonly used therapy to repair the ruptured regions, although some strategies like the Amniopatch appear promising (Deprest et al., 2011). Thus, there is a need to improve our understanding of this phenomenon, so that we can identify two groups of pPROM patients, those at risk for pPROM after infection and those at risk for non-infectious pPROM. Compounding this intricate challenge is that there are gaps in our fundamental knowledge as to how the fetal membranes weaken at the end of a normal pregnancy. Our lack of understanding of how normal membrane rupture occurs, impedes our ability to determine how this normal mechanism digresses during pPROM.

The importance of finding new therapeutic targets for the prevention of PTB, and also improving our understanding of basic parturition mechanisms, including rupture of the fetal membranes cannot be overstated. This is because much of the impact of PTB in the United States is borne by our minority populations. Americans who are members of racial and ethnic minority groups, (African Americans, American Indians and Alaska Natives, Asian Americans, Hispanics or Latinos, Native Hawaiians, and other Pacific Islanders), are more likely than Caucasians to have poor health and to die prematurely (CDC, 2020)[1]. States that have the highest rates of PTB disparity typically have large minority populations. Indeed, data from the March of Dimes mirrors this, showing that Hawai'i was ranked the 50th state in terms of PTB as a health disparity (March of Dimes Perstats[2]). The infant mortality rate is twice as high for Native Hawaiian mothers compared to whites and 43.9% of the cause of this infant mortality is PTB related (Hirai et al., 2013). Contributing to the lack of progress in Hawai'i is the lack of ethnic disaggregation, masking valuable information (Park et al., 2009; Tsark and Braun, 2009) as many established health disparities, including PTB, differentially affect ethnic groups within this population pool (Braun et al., 1996). In addition, we have no data on specific incidence of pPROM, versus other etiologies of PTB, although it is frequently seen in the clinic. It is likely that this is due to the general lack of focus on the importance of the fetal membranes in pregnancy outcomes, that is also seen in the other states. In Hawaii, like the rest of the United States, African American mothers have the highest rates of prematurity (13.8%) (March of Dimes Perstats: see text footnote 2). However, they only constitute 2.2% of the population (United States census data[3]). In other United States states African Americans constitute a much larger percentage of the population

Abbreviations: 15d-PGJ2, 15-deoxy-delta-12, 14-prostaglandin J2; a2V, V-ATPase; ADAMTS5, ADAM metallopeptidase with Thrombospondin type 1 motif 5; AEC, amnion epithelial cell; AMC, amnion mesenchymal cell; AP1, Activator protein 1; ATP, Adenosine tri-phosphate; CD, cluster differentiation; cffDNA, cell free fetal DNA; CLR, C-type lectin receptors; DAMP, Danger associated molecular pattern; DNGR1, Dendritic cell natural killer lectin group receptor-1; dsRNA, double stranded ribonucleic acid; ECM, Extracellular Matrix; ERK, extracellular-signal-regulated kinase; G-CSF, granulocyte colony stimulating factor; GIT2, ARF GTPase-activating protein; GSK, Glycogen synthase kinase 3; HBD2, b-defensin 2 HBD2; HKE, Heat killed *E. Coli*; HMGB1, high mobility group box 1; HMW, high molecular weight; HSP, Heat Shock protein; IFN, interferon; IL, Interleukin; iNOS, inducible nitric oxide synthase; IRF, IFN regulatory factors; IRF, interferon-regulatory factor; LMW, low molecular weight; LPS, Lipopolysaccharide; LRR, Leucine-rich repeats; MAL/TIRAP, MyD88 adaptor-like protein; MALP-2/FLS-1, diacyl lipopeptides; MAPK, mitogen activated protein kinase; MIP-1A, macrophage inflammatory protein; miRNA, micro ribonucleic acid; MMP, Matrix Metalloproteinase; mtDNA, mitochondrial deoxyribonucleic acid; MYD88, myeloid differentiation primary response protein 88; NF-kB, Nuclear Factor Kappa B; NLR, NOD-like receptors; NLRP3, LRR- and pyrin domain-containing protein 3; P2YR, P2 receptor; P2X7R, P2X purinoceptor 7 receptors; PAMP, Pattern associated molecular pattern; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; PGE2, prostaglandin E2; PGN, peptidoglycan; Poly I:C, polyinosinic-polycytidylic; pPROM, preterm premature rupture of the membranes; PRRs, Pattern recognition receptors; PTB, Preterm birth; PTL, Preterm labor; RAGE, advanced glycation end products; RANTES, regulated upon activation, normal T expressed and secreted; RD, repressor domain; RLR, RIG-1-like receptors; ROS, reactive oxygen species; SAA1, serum amyloid A1; SAP130, sin3A associated protein 3A; SASP, senescence-associated secretory phenotype; ssRNA, single stranded ribonucleic acid; ST2, suppression of tumorigenicity 2; TIM, T-cell immunoglobulin and mucin-containing domain-3; TIR, Toll/IL1 receptor; TLR, Toll-like receptors; TNF, tumor necrosis factor; TRAM, TIR domain-containing adapter molecule 2; TRIF, TIR domain-containing adaptor-inducing IFNβ TRIF; ZAM, zone of altered morphology.

[1]http://www.cdc.gov/omh/AMH/dbrf.htm

[2]http://www.marchofdimes.com/peristats/Peristats.aspx (accessed May 7, 2020).

[3]https://www.census.gov/quickfacts/fact/table/HI/PST045219# (accessed May 7, 2020).

and consistently have the highest prematurity rate (Schaaf et al., 2013). These studies are typically controlled for socioeconomic and demographic confounders and therefore to improve our understanding of the underlying cause, future studies need to focus on determining the risk factors for specific ethnic groups.

THE ONSET OF FETAL MEMBRANE WEAKENING MAY BE TRIGGERED BY CELLULAR STRESS

One of the fundamental remaining questions in the field of parturition research is how the tissues of pregnancy switch from a relatively "quiescent" state that favors the maintenance of the pregnancy, to one that is "reactive" in preparation for the delivery of the fetus. Animals other than humans and non-human primates experience a drop-in progesterone level but this does not appear to happen in the same way in humans (Menon et al., 2016a). In order to increase our understanding of the differences in the mechanism, studies have focused on areas of enquiry that may lead to "functional" progesterone withdrawal, such as the role of prostaglandin receptors (Nadeem et al., 2016; Patel et al., 2018) and the minutiae of inflammation control by cytokine cascades and specific transcription factors (Lappas et al., 2008; Paulesu et al., 2010). Understating the trigger for labor onset is important for us to decipher how this may deviate in patients with PTB. It is also important to know how this labor mechanism interfaces with the trigger for the initiation of fetal membrane remodeling and weakening, another pathway that is poorly understood.

The idea that cellular stress is the trigger for both fetal membrane weakening, and labor, has been gaining traction (Menon et al., 2016b). Suggested stressors for this mechanism have included; stretch/distension (**Figure 1**) of fetal membranes (Millar et al., 2000; Joyce et al., 2016) and myometrium (Waldorf et al., 2015), and also general hypoxia/oxidative stress in all of the tissues of pregnancy. Both of these stressors are known to increase in the human fetal membranes with gestational age or labor (Chai et al., 2012; Joyce et al., 2016), and also to stimulate inflammation (Kendal-Wright, 2007; Menon and Richardson, 2017). This has been demonstrated in all of the tissues of pregnancy and pregnancy complications result from the altered levels of cell stress in these tissues (Duhig et al., 2016). Indeed, several studies have shown that oxidative stress is linked to cell aging and senescence in cells of the amnion, directly leading to increased inflammation (Menon et al., 2017; Menon, 2019). It has also been shown to lead to epithelial to mesenchymal transition in the amnion, which can also play a role in the maintenance of the integrity of this tissue (Richardson et al., 2020). Other distinct types of cellular stress that have also been the subject of study in the human fetal membranes, including, Endoplasmic Reticulum Stress (Liong and Lappas, 2014) and Mitochondrial Stress (Than et al., 2009). In addition, cells can also respond to stress in a variety of way such as initiating, the heat shock response, the unfolding protein response or a DNA damage response (Fulda et al., 2010). Therefore, there are many specific pathways and mechanisms that constitute the wide umbrella term "cell stress," these should be further investigated to elucidate their contribution to the inflammation and cellular responses seen as the fetal membranes weaken.

One of the ways in which cell stress may lead to inflammation is through the production of Danger Associated Molecular Patters (DAMPs), also known as Alarmins (Sheller-Miller et al., 2017). These molecules typically have a different specific function during normal cellular activity, but when the cell detects a stress stimulus, they are activated now functioning to signal "the alarm.". Many different molecules are classified as DAMPs, including various heat shock proteins (HSP), extracellular matrix (ECM) breakdown products, and nucleic acid fragments (**Table 1**; Patel et al., 2018). DAMPs are already known to have a role in a wide range of other diseases with strong inflammatory signatures, such as, autoimmune diseases (Systemic Lupus Erythematosus, Rheumatoid Arthritis), Osteoarthritis, cardiovascular diseases, neurodegenerative diseases and cancer (Roh and Sohn, 2018). Here, they perpetuate a positive-feedback cycle of cellular damage, inflammation and then more cellular damage (Roh and Sohn, 2018). DAMPs are known to activate various Pattern Recognition Receptors (PRRs), including the Toll-like receptor (TLRs) family (Takeda et al., 2003; Kawai and Akira, 2010) and through these receptors, they can cause the activation of the pro-inflammatory transcription factor Nuclear Factor Kappa-B (NF-κB), changes in the levels of Matrix Metalloproteinases (MMP) and stimulate apoptosis (Roh and Sohn, 2018). Due to the large number of wide-ranging biomolecules acting as DAMPs (**Table 1**) and the large number of different receptors involved, they produce their effects by working through a complex number of distinct signaling pathways.

THE WEAKENING AND SUBSEQUENT RUPTURE OF THE HUMAN FETAL MEMBRANES IS DEPENDENT ON BIOPHYSICAL AND BIOCHEMICAL CHANGE

The fetal membranes are a multilayered structure composed of various cell types and associated ECM (**Figure 1**). The normal rupture of these membranes is currently thought to be the result of both physical forces and biochemical changes. The physical properties of fetal membrane strength are known to originate from the layer closest to the fluid and fetus (**Figure 1**), the amnion (Arikat et al., 2006). The strength of this tissue is undoubtedly derived from the combination of its layers working in concert. Some of this may come from the interface between the amnion and chorion. This region in the amnion is described as a spongy layer that is ECM rich (**Figure 1**), consisting of proteoglycans, glycoproteins and type III collagen (Strauss, 2013). The interface between this and the chorion consists of a gelatinous substance made up of hyaluronan, decorin, buglycan and collagen that mediates the separation of the amnion and chorion prior to fetal membrane rupture (Meinert et al., 2001, 2007). This separation is the first step in the weakening of the fetal membranes (Arikat et al., 2006). The chorion adheres to the decidua between

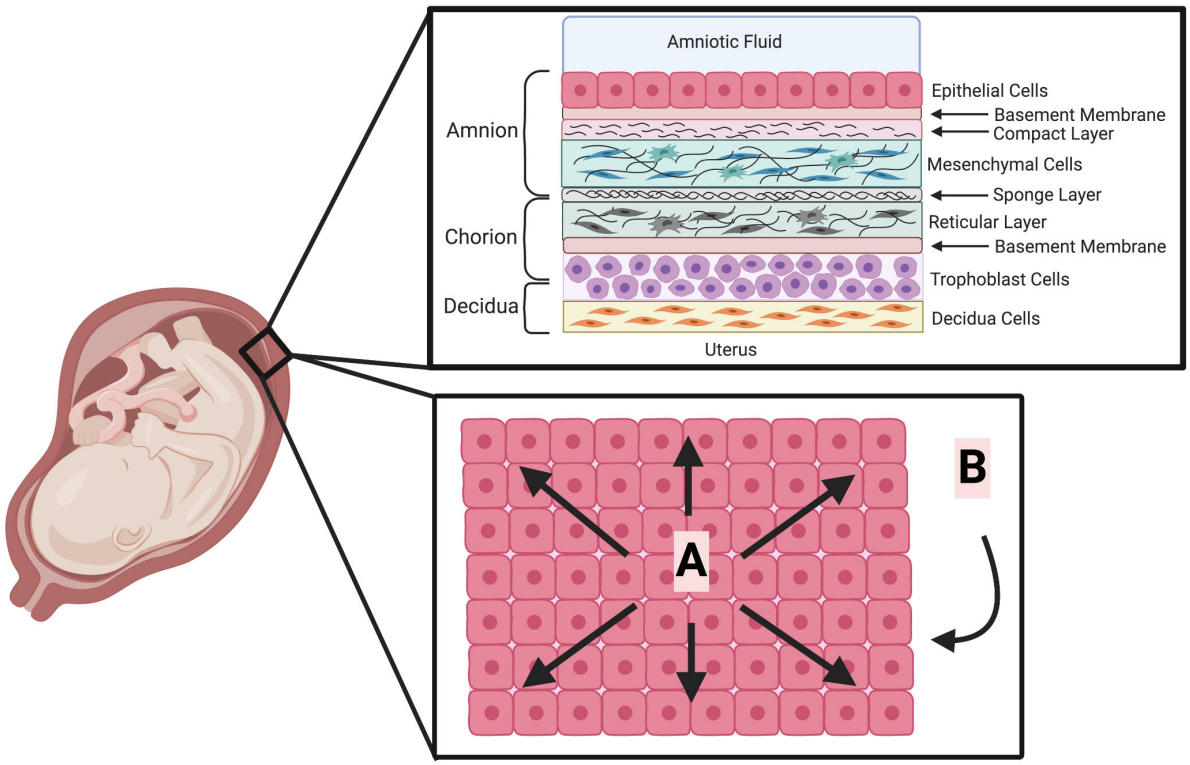


FIGURE 1 | The cellular structure of the fetal membranes and their biophysical forces. The human fetal membranes consist of a multi-layered structure which surround the fetus in utero. It is composed of the amnion, chorion and decidua (upper box). Biophysical forces are placed on these fetal membranes (lower box depicted looking down onto the apical surface of the amnion epithelium). **(A)** Linear physical forces due to multi-directional stretching of the fetal membranes adherent to the decidua. **(B)** Dynamic physical forces on the fetal membranes due to fetal movements, Braxton Hicks contractions and eventually labor. (This Figure was created using BioRender).

weeks 14 and 16 of pregnancy, by the degeneration of the capsular decidua and fusion of the chorion with the parietal decidua (Genbacev et al., 2015). Thus, this integration with the maternal tissues may also provide some strength to the tissue. However, the fetal membranes typically rupture in the region that is above the cervix (Malak and Bell, 1994; Strauss, 2013). *In vitro* it has been shown that after its separation from the amnion, this chorion layer is next to rupture (Arikat et al., 2006). Therefore, as the amnion layer is last to rupture in this sequence, after a notable period of deformation, it is widely accepted that the ECM rich compact layer containing amnion mesenchymal cells (AMC) (**Figure 1**) accounts for the strength and maintains the integrity of this tissue (Arikat et al., 2006).

An increase in apoptosis (Fortunado et al., 2000; Hsu et al., 2000; Kumagai et al., 2001) and changes in the levels of MMPs (Cockle et al., 2007) are central to the biochemical component of the changes that occur in the fetal membranes before their rupture. Although cell death in the form of apoptosis is recognized as important for the weakening process, it is thought that these cells can also die through autophagy (Shen et al., 2008; Mi et al., 2017) and perhaps necrosis (Menon and Richardson, 2017), as both of these forms of cell death are also known to be the result of cell stress (Fulda et al., 2010). In addition, it is known that necrosis can occur as the result of TLR activation in other cells (Meylan and Tschopp, 2005). Although cellular survival is obviously directly linked to the maintenance of the integrity of the amnion, its physical strength is dependent on the synthesis and degradation of the components of the ECM (El Khwad et al., 2005; Anum et al., 2009) controlled by resident cells (Parry and Strauss, 1988). Indeed, women with connective tissue disorders and related diseases are at an increased risk for complications during pregnancy, including pPROM (Anum et al., 2009). Support for this mechanism has come from several research groups as they have biochemically and mechanically identified a "zone of altered morphology" (ZAM) in the human fetal membranes (McParland et al., 2003; El Khwad et al., 2005; Osman et al., 2006; Reti et al., 2007). The ZAM constitutes a discrete zone of weakness overlying the cervix characterized by several features; an increased thickness and swelling of the connective tissue layer, a reduction in both the cytotrophoblast and decidual layers, and a reduced overall thickness of the supracervical membranes that exhibits increased ECM remodeling (Lappas et al., 2008), and apoptosis (Shen et al., 2008). It is also known that inflammation in the form of increased cytokine secretion and signaling are

TABLE 1 | Summary of key Danger Associated Molecular Patterns, their receptors and mechanisms.

Origin	DAMP	Receptor	**Function** (standard font = pro-inflammatory; italics = anti-inflammatory)	References
Extracellular Matrix	Aggrecan 32 mer fragment	TLR2	(iNOS), CCL2, IL-1α, IL-6, MMP12 MMP13 and ADAMTS5	Stevens et al., 2008
	Biglycan	TLR2/4	Increases levels of reactive oxygen species, CXCL-1, CCL2 and HSP70, and activates NLRP3 inflammasome by Caspase-1 and the maturation of IL-1β *Activating a TLR co-adaptor that activates IFN1 signaling*	Frey et al., 2013; Schaefer, 2014; Roedig et al., 2019
	Decorin	TLR2/4	Decreases TGFβ1 and IL-10 and increases levels of apoptosis	Merline et al., 2011
	*Fibronectin	TLR2/4	Promotes pro-inflammatory mediators and phagocytosis by macrophages	Haruta et al., 2013; Fei et al., 2018
	Fibrinogen	TLR4	Activation of monocytes	Al-Ofi et al., 2014
	LMW-HA	TLR2/4	Activates NLRP3 inflammasome by Caspase-1 and the maturation of IL-1β. Activates NF-kB	Merline et al., 2011
	HMW-HA	TLR2	*Activates a TLR co-adaptor that activates IFN1 signaling*	Scheibner et al., 2006; Frey et al., 2013
	Heparin sulfate	TLR4, RAGE	Activation of NF-kB	Xu et al., 2011
	Tenascin C	TLR4	Synthesis of pro-inflammatory cytokines	Midwood et al., 2009
	Versican	TLR2/4 CD14	IL-6, IL-1β, Il-12 and CCL2 production *Increasing IL-6 (anti-inflammatory pathways), IL-10*	Wight et al., 2014
Cytosolic	ATP	P2XR P2YR	Attracts macrophages by inflammasome activation MAPK wound healing response	Venereau et al., 2015
	Cyclophilin	CD 147	Chemotaxis and the production of pro-inflammatory factors	Burkrinsky, 2014
	F-actin	DNGR1	DNGR1 recognizes the released F-actin, which causes the uptake of damaged or dead cells	Brown, 2012
	Heat Shock Protein	TLR2/4	MyD88 dependent activation of NF-kB	Tolle and Standiford, 2013; Relja et al., 2018
	S100 proteins	TLR4, CD147 RAGE	Leads to apoptosis and activates ERK and NF-kB or AP1	Ghavami et al., 2010; Xia et al., 2018
	*Soluble amyloid beta	TLR2/4	Enhanced TNF driven inflammation	Wang et al., 2019
	Uric Acid	NLRP3	Inflammasome activation and induction of IL-1β maturation	Braga et al., 2017
Mitochondrial	mtDNA	TLR9	p38 MAPK and NF-kB activation	Zhang et al., 2010; Zhang et al., 2014; Magna and Pisetsky, 2016; Bao et al., 2016
Nuclear	*Cell free DNA	TLR9	Activation of NF-kB and AP1	Magna and Pisetsky, 2016
	Circulating Histones	TLR9	Inflammation through the activation of NF-kB	Huang et al., 2011; Kawai et al., 2016
	Extracellular self RNA	TLR7 TLR3	Sensitizes other TLR working synergistically with their other ligands MAPK, NF-κB, and IRF-5/7 pathways through MyD88 signaling	Karikó et al., 2004; Cavassani et al., 2008; Thompson et al., 2011; Noll et al., 2017; Petes et al., 2017
	*HMGB1	TLR2 TLR4 TLR9	Activation of NF-kB, and MAPK signaling though ERK and p38, release of MMPs	Qin et al., 2006; Nie et al., 2016
				Menon et al., 2011; Bredeson et al., 2014; Plazyo et al., 2016
	IL-1α	IL-1R	MAPK signaling and NF-kB activation	Betheloot and Latz, 2017
	IL-33	ST2	NF-kB activation and TNF production	Isnadi et al., 2018
	SAP130	Mincle	Triggering pro-inflammatory cytokine secretion	Zhou et al., 2016; Patkin et al., 2017

** denotes has been studied in the fetal membranes or cells of the fetal membranes. ADAMTS5, ADAM metallopeptidase with Thrombospondin type 1 motif 5; AP-1, Activator protein 1; CD, Cluster Differentiation; DNGR1, Dendritic cell natural killer lectin group receptor-1; ERK, extracellular-signal-regulated kinase; HSP, Heat Shock protein; IFN1, Interferon; IL, Interleukin; iNOS, Inducible Nitric Oxide; IRF, Interferon regulatory factors; MMP, Matrix Metalloproteinase; MyD88, myeloid differentiation primary response protein 88; NF-kB, Nuclear Factor Kappa B; NLRP3, LRR- and pyrin domain-containing protein 3; RAGE, advanced glycation end products; ST2, suppression of tumorigenicity 2; TGF, Transforming growth factor; TLR, Toll-like receptor; TNF, Tumor Necrosis Factor.*

also involved in the initiation and progression of membrane rupture both at term and preterm. This is particularly evident when associated with intrauterine infection and chorioamnionitis (Bowen et al., 2002). However, it is important to note that inflammation in the absence of infection, in the form of what has been coined "sterile inflammation," leads to pPROM and normal rupture of the membranes (Shim et al., 2004). Roles for several key pro-inflammatory cytokines; Interleukin 1β (IL-1β), IL-6, IL-8, and Tumor Necrosis Factor-α (TNF-α) in parturition are apparent. Their increase in abundance in gestationally advanced fetal membranes is not only associated with labor (Keelan et al., 1999) but they have also all been demonstrated to independently increase the synthesis of MMPs (Bowen et al., 2002). Additionally, many of these cytokines can

cause the translocation of the pro-inflammatory transcription factor NF-κB thus leading to further increases in inflammatory mediators. This provides a pathway of pro-inflammatory self-induction (Christiaens et al., 2008) that is thought to terminate with delivery of the fetus. This can be exemplified by the chemokine IL-8, which leads to the increased infiltration of polymorphonuclear leukocytes, which can further contribute to the increase in inflammation in a feed-forward manner and can lead to birth. In further support of a central role for cytokine-induced cascades in membrane weakening, TNF-α and IL-1β have been shown to directly cause significant weakening of fetal membranes, inducing the biochemical markers characteristic of the ZAM (Kumar et al., 2006).

In addition to the polymorphonuclear leukocytes that are attracted to the tissue by chemokines, an infectious inflammatory response leads also leads to the recruitment of macrophages. These produce cytokines, MMPs, and prostaglandins, which increase the risk of pPROM (Parry and Strauss, 1988). In addition, stimulated monocytes in human chorionic cells produce the inflammatory cytokines IL-1α and TNFα, which result in the increased expression of MMP-1 and MMP-3 (Katsura et al., 1989; So et al., 1992). In fetal membranes with chorioamionitis, adhesive granulocytes have also been noted adjacent to apoptotic amnion epithelial cells (AECs) near the rupture site (Leppert et al., 1996). Together these data illustrate how immune cells promote cellular changes within the fetal membranes by driving inflammation, and breaking down ECM through the production of MMPs, predisposing the tissue for rupture. However, more recently it has been demonstrated that immune cells may also have fetal membrane healing properties through the migration of macrophages from the amniotic fluid to a rupture site in the amnion (Mogami et al., 2017). These cells were seen to induce wound healing by secreting IL-1β and TNFα and stimulating epithelial to mesenchymal transition (Mogami et al., 2017).

It is thought that the forces produced by the cell stressor distension, may be the link between the biochemical and biophysical changes seen in the fetal membranes toward term. This was originally based on the observation that human pregnancies with more than one fetus often result in premature delivery (Keith and Oleszczuk, 2002). The insertion and subsequent slow inflation of a balloon above the cervix in humans is also known to induce labor (Manabe et al., 1985). This led to the study of the distension of the uterus to discern the resultant biochemical changes and how they might lead to the activation of uterine contraction (Shynlova et al., 2009). Less work has been performed studying the effect of distension on the fetal membranes although it has been clearly shown that they are massively stretched *in vivo* at term (Millar et al., 2000; Joyce et al., 2016). It is assumed that this is the result of the combination of their adherence to the uterine wall and the termination of cellular proliferation, halting their further growth, at the beginning of the third trimester (**Figure 1**). Work performed stretching both the uterus, pieces of fetal membranes (Nemeth et al., 2000) or cells of the amnion (Kendal-Wright et al., 2008, 2010) show that this stimulus is able to induce pro-inflammatory cytokine production and secretion, and can also regulate apoptosis (Kendal-Wright et al., 2008; Poženel et al., 2019). Thus, the distension of the fetal membranes in normal term pregnancies and its over distension in PTB, can lead to its inflammatory signature. This distension also constitutes a significant source of cellular stress through physical strain. Interestingly, our recently collected data confirms that cellular distension of cells of the amnion *in vitro* can indeed act as a cell stressor, increasing the secretion of the DAMP, High mobility group box 1 (HMGB1) (Norman Ing et al., 2019).

DANGER ASSOCIATED MOLECULAR PATTERNS ARE A LARGE GROUP OF BIOMOLECULES WITH DISTINCT CELLULAR COMPARTMENTALIZATION

Danger Associated Molecular Patterns are a wide-ranging group of biomolecules, originating from various cellular compartments. These molecules were classified as DAMPs when released, activated or secreted in response to tissue injury, and by damaged or dying cells (Schaefer, 2014). They can originate from nuclear or intracellular location, or cleaved from ECM. They have a wide range of effects resultant from their interaction with PRR on both immune cells and endogenous cells of organs (**Table 1**). The intention here is not to discuss an exhaustive list of all those that have been identified to date, but to briefly highlight the distinct origins of DAMPs and discuss what is known about them in the fetal membranes (**Table 1**).

The ECM has an important role in shaping the innate immune response, it is dynamic, not simply a static network that provides tissue integrity and strength. The majority of DAMPs coming from the ECM are derived from proteoglycan or glycoprotein (**Table 1**), and are typically released by the cleavage by MMPs, Hyaluronidase, and Heparanase (Gaudet and Popovich, 2014). However, they can also be *de novo* synthesized or released by unfolding due to mechanical stimulation (Smith et al., 2007). When released, they function to trigger sterile inflammation or prolong pathogen-induced responses by "fine-tuning" the production of inflammatory mediators (Frevert et al., 2018). Some are known to promote inflammation, whereas others are also anti-inflammatory (Frevert et al., 2018). These diverging roles are dependent on the activation of specific signaling profiles working through specific PRRs. Thus, these DAMPs work to modulate inflammation by their interaction with a range of receptors including; TLRs (TLR2 and TLR4), RIG-1-like receptors (RLRs), NOD-like receptors (NLRs), receptor for advanced glycation end products (RAGE), integrins and cluster differentiation 44 (CD44). Although their role has not been directly studied in the fetal membranes, it is reasonable, given that the amnion that provides the strength of the tissue is ECM rich (**Figure 1**) and is subject to cell stress in the form of distension toward term, that these molecules could have a key role in driving

the weakening mechanisms of this tissue toward the end of gestation.

A wide range of DAMPs from various intracellular origins have also been characterized (**Table 1**). They can be cytosolic, endoplasmic, or they can also be released from granules or the plasma membrane. However, one of the most important unifying characteristics of this group of DAMPs is that when "activated" by cell stress, they typically change cellular compartment and lead to differential signaling events, compared to those seen during their normal biological role in "unstressed" conditions. In addition, various proteins and nucleic acids from the nucleus and mitochondria have been widely studied for their DAMP roles (**Table 1**). This group of DAMPs include but are not limited to; HMGB1, circulating histones, various interleukins, Free DNA, mitochondrial DNA, and self-extracellular RNA. The variety of DAMPs provide a range of receptor specificity and pro/anti-inflammatory functionality (**Table 1**).

DANGER ASSOCIATED MOLECULAR PATTERNS AND PATHOGEN ASSOCIATED MOLECULAR PATTERNS ACTIVATE PATTERN RECOGNITION RECEPTORS

It is clear that DAMPs cause inflammation in other tissues, therefore we believe they could be the key regulators that begin the weakening of the fetal membranes by driving inflammation during this process too. They elicit their effects through numerous receptors known as PRRs. One of the largest groups of receptors that belong to this group is the TLRs. They are a distinct class of germline-encoded PPRs that initiate the innate immune response for the initial detection of a pathogen (West et al., 2006). They are located on cell surfaces or within endosomes and generally have a conserved function to protect against pathogens via activation of downstream signaling pathways (Kawai and Akira, 2010). Several roles of TLRs have been identified including the clearance of pathogenic microbes, protection of endogenous threats, and regulation of the innate and adaptive immune response (Hug et al., 2018). There are ten TLR isoforms (TLR1–10) in humans that are expressed on immune and non-immune cells including macrophages, fibroblasts, epithelial cells, and endothelial cells (Kumar et al., 2009). TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface, whereas TLR3, TLR7, TLR8, and TLR9 are expressed in endosomes (Takeda, 2004; Kumar et al., 2009). TLR10 is a distinct receptor, in that it is the only TLR known to act as inhibitory protein through the induction of anti-inflammatory cytokine IL-1Ra (Oosting et al., 2014).

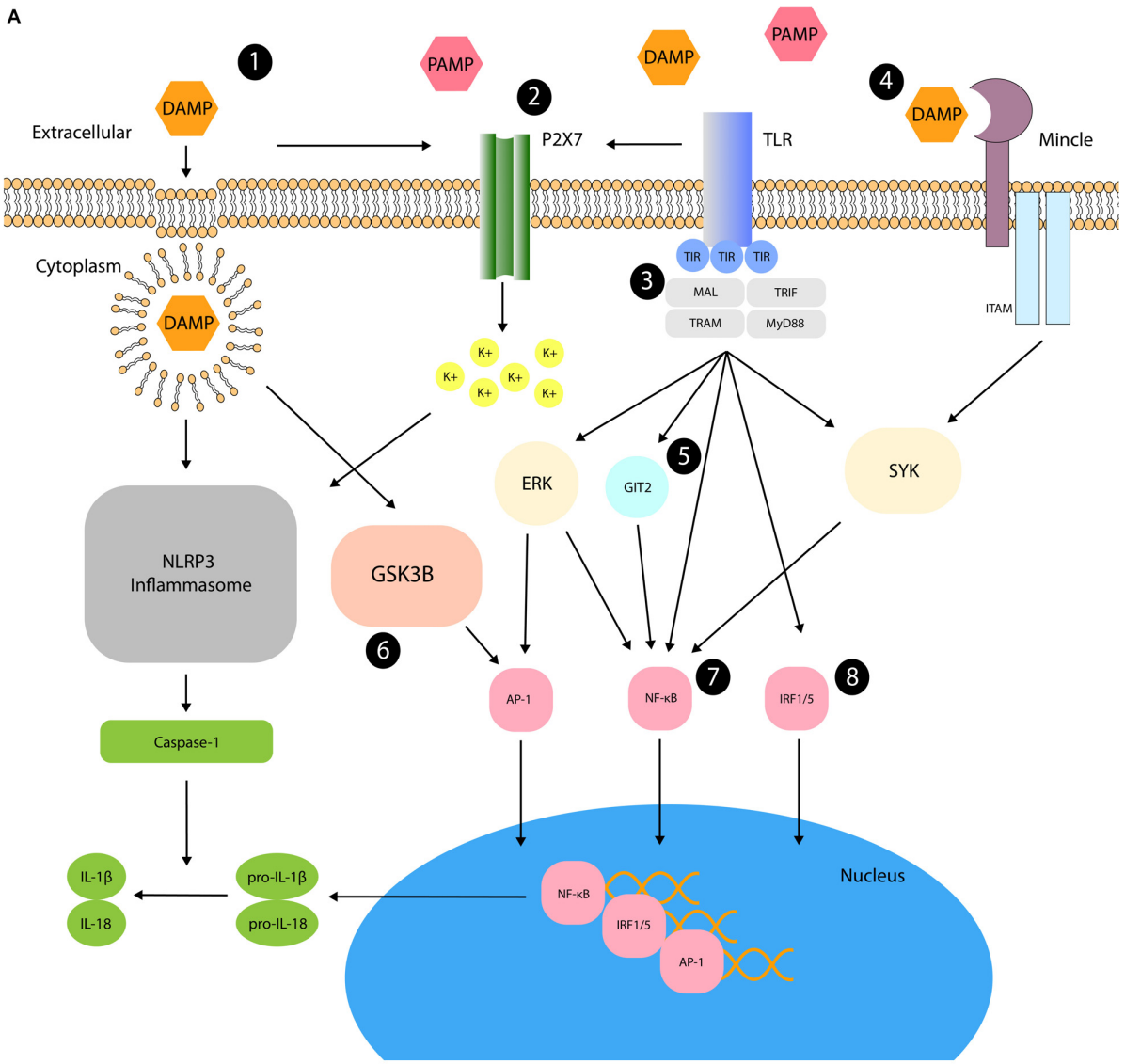
The cell surface TLRs mainly recognize PAMPs generated from cell wall components and flagellin from gram positive and gram-negative bacteria, yeast, and fungi (Chaturvedi and Pierce, 2009). Interestingly, the recognition of DAMPs by these cell surface TLRs are shown to require different co-receptors and accessory molecules to the PAMPs. The endogenous ligands of these receptors appear to be limited, perhaps to help constrain TLR responses so that they can sufficiently function for pathogenic recognition, without causing detrimental responses to the host (Miyake, 2007). The intracellular TLRs (TLR3, 7, 8, 9) function within endolysosomal compartments and detect foreign nucleic acids that are signatures often belonging to invading viruses and microbes (Blasius and Beutler, 2010). Generally, it is thought that the endosomal TLRs are able to distinguish between host and foreign nucleic acids, although there is mounting evidence that some TLRs may not have this ability, and cannot discriminate between the nucleic acid molecules of host and microbial origin (Blasius and Beutler, 2010). It is known that endogenous mRNA and RNA from necrotic cells can also activate the intracellular TLR mediated pathway (Karikó et al., 2004; Cavassani et al., 2008; Thompson et al., 2011), resulting in an antiviral and pro-inflammatory response via Interferon (IFN) and cytokine induction (Perales-Linares and Navas-Martin, 2013).

Although the family of TLRs were originally identified for their abilities to recognize and mediate signaling pathways for a variety of microbial components (Poltorak, 1998; Takeuchi et al., 1999, 2001, 2002; Hemmi et al., 2000; Alexopoulou et al., 2001; Hayashi et al., 2001; Lund et al., 2004) many studies have shown that they are important in the detection of endogenous DAMP molecules (**Table 1**; Beg, 2002; Wallin et al., 2002). This is where the function of PAMPs and DAMPs differ, as DAMPs are also necessary for tissue repair. Further exploration into the exogenous (PAMP) and endogenous (DAMP) ligand activation of the TLR family may provide new and effective therapies to mediate the negative effects of TLR-driven inflammation.

Several PRRs and co-receptors other than the family of TLR have also been shown to be important for the function of DAMPs and PAMPs (Takeuchi and Akira, 2010). These include, but are not limited to, RLRs, NLRs, RAGE, C-type lectin receptors (CLRs), P2X purinoceptor 7 receptors (P2X7Rs), LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome, and distinct members of the cluster differentiation receptor family. Similarly, to TLRs, all of these receptors are responsible for triggering distinct inflammatory cascades of the immune response. Some literature suggests that the intervention of DAMP and PAMP driven inflammation through these receptors, and their specific signaling signatures, may ablate the negative effects of associated pathologies (**Figure 2**).

DANGER ASSOCIATED MOLECULAR PATTERNS AND PATTERN ASSOCIATED MOLECULAR PATTERNS IN THE FETAL MEMBRANES

There is a growing body of evidence demonstrating that DAMPs and PAMPs work via a similar set of PRRs to guide the responses of innate immune system. However, much of what we understand about the potential of DAMPs to cause fetal membrane weakening and pPROM comes from work studying



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	Target	Intervention(s)	Reference(s)
1.	DAMP	Anti-HMGB1 antibody; DNA-binding A box	Yang, 2004
2.	P2X7	Progesterone (P4); prolactin; oATP; KN-62	Maneta, 2015
3.	TLR	Anti-TLR monoclonal antibody; neutralizing antibody; soluble RAGE; Tollip; naloxone; Pelliono 1; Tim3	Waldorf, 2008; Agrawal, 2013; Chin, 2016; Li 2015; Lim, 2018a; Wang, 2015; Wong, 2018
4.	Mincle	siRNA (siCLEC4E)	Lim, 2019a
5.	GIT2	siRNA (siGIT2)	Lim, 2019b
6.	GSK3β	CHIR99021	Lim, 2015
7.	NF-κB	N4-[2-(4-phenoxyphenyl)ethyl]-4,6-quinazolinediamine (QNZ); 15-deoxy-delta-12, 14-prostaglandin J2 (15d-PGJ2)	Maneta, 2015; Pirianov, 2006
8.	Interferon	siRNA (siIRF5, siIRF1)	Lim, 2016, 2018b

FIGURE 2 | DAMP/PAMP PPR pathway manipulation. **(A)** TLR, Mincle, and P2X7 receptor activation regulated by DAMP and PAMP signaling have been shown to stimulate downstream pathways involving various modulatory proteins (ERK, GSK3B, GIT2, SYK). This may activate AP-1, NFkB, IRF1/5, or inflammasome activation that leads to induction of pro-inflammatory cytokines. Studies have demonstrated different areas of modulation (1–8) that can manipulate DAMP/PAMP mediated inflammation associated with PTB. **(B)** The supporting table lists several interventions that demonstrate the manipulation of the DAMP/PAMP mediated pathways that correspond to the different areas of modulation.

the effect of PAMPs in this and other tissues. However, it cannot be assumed that the signaling events and downstream outcomes between these two groups of biomolecules (DAMPs and PAMPs), are identical, even during their activation of the same receptor. Regardless, work on the role of PAMPs in the fetal membranes has been able to provide us with insights as to how DAMPs may also function, providing foundational data that can guide our understanding of their potential as directors of fetal membrane weakening. There is a clear link between fetal membrane weakening and PAMP driven inflammation in PTB with pPROM. Indeed, TLR-mediated PTB in response to PAMPs have been studied and extensively reviewed elsewhere (Thaxton et al., 2010). Thus, the intention of this review is to discuss the work most relevant to and that was performed in the fetal membranes.

PAMP Activation of Inflammation in the Fetal Membranes

Inflammation initiated by bacterial infection causes PTB by preterm labor and pPROM. The initiation of this inflammation is caused by different PAMPs upon their interaction with their specific TLR; for example, lipopolysaccharide (LPS) with TLR4. To determine the role of PAMP ligands specifically in fetal membranes, numerous *ex-vivo* tissue explant studies have been completed. Under normal physiological conditions the fetal membranes express all of the TLR isoforms (1-10) that could be activated and lead to specific pro-inflammatory signatures. Indeed, the treatment of fetal membrane explants with bacterial TLR agonists; peptidoglycan (TLR2), LPS (TLR4), and flagellin (TLR5) all produce a pro-inflammatory response with increased production of various cytokines, including; IL-1β, IL-6, IL-8, IL-10, granulocyte colony stimulating factor (G-CSF), macrophage inflammatory protein (MIP-1A) and Regulated upon Activation Normal T Expressed and Secreted (RANTES) (Hoang et al., 2014).

Not only do bacterial ligands lead to infection driven inflammation but they also lead to increases in the receptors that detect them. This was demonstrated in normal human fetal membranes, where the levels of TLR expression changed upon exposure to various bacteria associated with PTB pPROM. Specifically, treatment with the bacterial stimuli *Mycoplasma hominis* lead to increased expression of TLR 4, 6, and 8. Increased expression of TLR8 was also seen with *Ureaplasma parvum* and increased expression of TLR7 upon treatment with *Porphyromonas gingivalis*. Interestingly, gram negative *E. Coli* significantly decreased TLR10 expression rather than increase TLR expression as demonstrated by the other bacterial stimuli. This is of note as TLR10 is still considered an orphan receptor and therefore its function is not well understood. These data suggest that fetal membranes vary their TLR expression levels upon treatment with bacterial ligands. This observation may be used in the future to indicate the severity of infection and differences in the receptor expression level will likely further direct the magnitude of an inflammatory immune response (Abrahams et al., 2013). In further support of the bacterial ligands affecting the levels of the TLRs, fetal membranes with chorioamnionitis, have also been shown to have differential expression of TLRs. The increased expression of *TLR1* at the gene level in preterm fetal membranes with histological chorioamnionitis has been described (Waring et al., 2015), as have increases in TLR1, TLR2, TLR4, and TLR6 in chorioamniotic fetal membranes (Moco et al., 2013). Together these data suggest a role for these receptors not only in the surveillance of bacteria in this tissue but also that they adapt to the bacterial challenge.

Chorioamnionitis is not the only route to bacterial infection in the fetal membranes. Periodontal disease is a chronic inflammatory disease caused by multiple strains of bacteria, several of which have been detected in amniotic fluid, placenta and fetal membranes. It is now accepted that there is a link between these bacterial and several pregnancy complications including PTB. *P. gingivalis* has been detected in chorionic tissue and LPS derived from it, used to treat chorion derived cells. This resulted in increased expression of TLR2 causing the increased production of IL-6 and IL-8. To further study this relationship, TLR-2 gene-silenced chorionic derived cells demonstrated a reduction in IL-6 and IL-8 secretion. Taken together this suggests an important role for TLR2 in periodontic bacterial signaling in the fetal membranes (Hasegawa-Nakamura et al., 2011). In support of this, the periodontal bacteria *Fusobacterium nucleatum* has also been detected in chorionic tissue from high-risk pregnant women. Similarly, to the previous study, it was then used to treat chorionic cells where it was also able to increase inflammation by increasing IL-6 and corticotrophin-releasing hormone (CRH) secretion via TLR2 and TLR4 activation (Tateishi et al., 2012). These data indicate that periodontal bacteria act similarly to other PAMPs in these tissues as they find their way into the amniotic fluid, placenta and fetal membranes, where they activate the TLR system and downstream inflammation.

Although humans are not thought to experience the sharp drop in progesterone that is thought to initiate the onset of parturition in other animals (Menon et al., 2016a), its administration is used to maintain pregnancy and lower the risk of PTB (Chenung et al., 2020) as it is thought that humans may experience a "functional" progesterone withdrawal (Nadeem et al., 2016). Progesterone (P4) has been shown to have a protective role in fetal membranes by reducing pro-inflammatory cytokine production upon LPS treatment (Flores-Espinosa et al., 2014). It does this in part by significantly reducing TLR4 expression upon LPS treatment. This directly results in the reduced secretion of the pro-inflammatory cytokines TNFα, IL-6, and β-defensin 2 (HBD2) (Flores-Espinosa et al., 2014). Therefore, this intriguing mechanism could be a way for progesterone to decrease the ability of this tissue to detect DAMPs until its functional levels decrease toward the end of gestation.

Animal models have also been used to determine how PAMPs induce inflammation leading to different pregnancy complications. They have also provided models of PTB that can be used to test novel therapeutic targets. In humans, the histological assessment of preterm fetal membranes detected TLR4 expression in the fundus and low segment suggesting a role in PTB (Choi et al., 2012). This receptor's role was further

investigated in an infection, LPS-induced PTB mouse model. The TLR4 antagonist (+)-naloxone, was able to suppress the expression the inflammatory cytokines IL-1β, IL-6, TNFα, and IL-10 and prevent PTB. Another study also established the role for TLR4 in PTB by using a mouse model by inducing infection with an LPS variant, heat killed *E. Coli* (HKE). HKE treatment induced PTB in all TLR4 normal mice compared to none of the TLR4 mutant mice. In addition, another study induced PTB in CD1 mice by administering intrauterine injections of saline, peptidoglycan (PGN, TLR2 agonist) or polyinosinic-polycytidylic acid (Poly I:C, TLR3 agonist) (Jaiswal et al., 2013). The regulation of a2 isoform of V-ATPase (a2V) has a role in pregnancy and was assessed post exposure to these two ligands, PGN and Poly I:C. This led to significantly decreased expression of a2V in the fetal membranes and play a role in the induction of PTB in mice (Jaiswal et al., 2013).

Collectively, several mouse studies have provided evidence that demonstrate the importance of bacterial PAMP/TLR driven inflammation to cause PTB. Providing model systems that can be continued to be used to study future therapies to address PTB by these pathways. Indeed, models activating TLRs by ligand injection (Kaga et al., 1996; Wang and Hirsch, 2003) or placental stimulation (Liu et al., 2007; Koga et al., 2009) are also models for PTB.

PAMP Stimulation of Amnion Epithelial Cells Isolated From Fetal Membranes

Human AECs form the most superficial layer of the fetal membranes functioning as an immunological barrier for the fetus against intra-amniotic infection. Their response to pregnancy specific PAMP ligands has been studied to help us understand the contribution of these cells to inflammation in the fetal membranes but also to their production of pro-inflammatory signaling molecules that may also communicate with the growing fetus. Under normal physiological conditions, these cells express TLR 1-10 and can be activated by the PAMPs that reach them. TLR5 and TLR 2/6 activation by PAMP the ligands, Flagellin and macrophage-activating lipopeptide-2 (MALP-2), respectively, significantly increase the secretion of IL-6 and IL-8 (Gillaux et al., 2011). They also cause the nuclear translocation of NF-κB subunit p65, thus inducing a pro-inflammatory response. The activation of TLR4 by LPS, however, has also been demonstrated to induce apoptosis and decrease cell viability of these cells (Gillaux et al., 2011). However, the activation of this receptor also increases Transforming Growth factor beta 1 (TGFβ1) and prostaglandin E2 (PGE2), TNFα and IL-1β production (Motedayyen et al., 2019), which has been confirmed for TGFβ1 and PGE2 but not for IL-1β, IL-6, and IL-10 (Taheri et al., 2018). Taken together these data suggest that not only does the activation of this PAMP TLR mechanism mediate an immunological response to PAMPs in AEC, but it could contribute to the inflammation seen in the fetal membranes with PTB and may also signal to the fetus when an infection was detected. It is possible that AMC respond similarly to AEC to PAMP stimulation, however, there has been a lack of focus on this system in AMC. In addition, differences in the fundamental functions of these cells in the fetal membranes make it difficult to evaluate similarities between them. Thus, it is challenging to know how similar the pathway signaling is in this cell type, that functions to maintain the integrity of ECM in the fetal membranes.

PAMP Stimulation of Amnion Mesenchymal Cells Isolated From Fetal Membranes

Human AMCs are located within the ECM rich layer of the amnion that is closest to the chorion in the of fetal membranes (**Figure 1**). This layer is responsible for the maintenance of the strength and integrity of the whole tissue. Our work has shown that the human AMCs isolated from human fetal membranes express all ten TLR isoforms (Sato et al., 2016). This suggests that similarly to AECs, AMC are able to detect PAMPs and produce a pro-inflammatory signature. However, as their location and function are different to that of AEC, the downstream consequences may also be different. We have also been able to show that indeed the TLR2/6 ligand MALP-2, could increase the pro-inflammatory cytokines and NF-κB translocation, it did not increase levels of apoptosis.

Viral PAMP Activation of the Fetal Membranes

In addition to bacterially derived PAMPs, viral infection also elicits an immune response through the activation of TLRs. This is typically through the release and subsequent detection of viral nucleic acids. TLR 3, 7, 8, and 9 are all nucleic acid ligand response receptors that are activated upon exposure to viral nucleic acid ligands. Thus, fetal membrane explants treated with viral double stranded RNA (dsRNA) and viral single stranded RNA (ssRNA) activate TLR 3 and TLR8, respectively, and induce downstream pro-inflammatory cytokine production that result in two different distinct antiviral response profiles (Bakaysa et al., 2014). Those explants treated with Poly (I:C) (TLR3), or the viral ligands, imiquimod (TLR7), and ssRNA40 (TLR8) also caused the production of pro-inflammatory cytokines IL-6 and IL-8 (Bryant et al., 2017). Although there is little data on the response of the fetal membranes to viral PAMPs, these data support the idea that all of the viral, as well as bacterial TLR receptors are able to be activated on cells of the fetal membranes and that they could result in inflammation, potentially leading to fetal membrane weakening and rupture.

DAMPS and Fetal Membranes

Work studying the direct effect of DAMPs or DAMP production by the fetal membranes is limited. Indeed, so far to our knowledge only four have been directly tested in the fetal membranes (**Table 1**). However, others that have also been shown to play a role in pregnancy are, HMGB1, cell-free fetal DNA (cffDNA), uric acid and IL-1. Little is known about the specific roles of any of these molecules in fetal membranes, and their potential as novel therapeutic targets and biomarkers in the pathologies of pregnancy.

High mobility group box 1 is the most comprehensively studied DAMP in the fetal membranes and has been shown to be a critical regulator throughout different stages of pregnancy. It activates TLRs to initiate an inflammatory immune response in a sterile environment, but it is also released by PAMP stimulation of TLRs. This was confirmed by its presence in the Amniotic fluid from preterm mothers with damaged fetal membranes due to intra-amniotic infection (Baumbusch et al., 2016). Its increase in levels also correlate with increases in IL-6 and with infection in amniotic fluid but its does not seem to increase with gestational age in this compartment. HMGB1 levels are also elevated in chorioamniotic membrane extracts from preterm labor compared to term labor (Plazyo et al., 2016) and it has been seen that this increase contributes to increased expression of pro-inflammatory cytokines (Plazyo et al., 2016). It also increases *MMP-9* gene expression and active-MMP-9 levels in chorioamniotic fetal membrane explants, compared to control treated fetal membranes (Plazyo et al., 2016). These data suggest that HMGB1 induction of MMP-9 could directly promote fetal membrane weakening. Pregnant mice treated with intra-amniotic injections of HMGB1 resulted in increased pup mortality, PTL and PTB compared to pregnant mice treated with PBS (Gomez-Lopez et al., 2016). This study did not asses the role of HMGB1 in the fetal membranes, but these data suggest HMGB1 has an important role in promoting PTB. Fetal membranes treated with exogenous HMGB1 increase TLR2 and TLR4 expression and activation of the inflammatory immune response regulator, p38 Mitogen Activated Protein Kinases (MAPK) which also leads to a senescent phenotype consistent with a sterile inflammatory response (Bredeson et al., 2014). Moreover, term fetal membranes have a phenotype consistent with senescent cells, reduced telomeres, increased activation of p38 MAPK and increased in senescence-associated beta (SA-B) galactosidase and senescence-associated secretory phenotype (SASP) gene expression found a senescent phenotype (Menon et al., 2016b). HMGB1, oxidative stress and apoptosis is also increased in normal fetal membrane explants exposed to cigarette smoke extract compared to control treated fetal membrane explants (Menon et al., 2011). In a study that also linked HMGB1 with fetal membrane weakening, fetal membranes from women who had preterm labor or pPROM were found to have increased levels of HMGB1 in their serum (Qiu et al., 2017). This suggests that HMGB1 could also be a potential biomarker for PTB. Immunofluorescent labeling of fetal membranes delivered preterm with chorioamnionitis also showed HMGB1 localized AECs, with more diffuse labeling in the myofibroblast and macrophages compared to term fetal membranes delivered at term (Romero et al., 2011). Interestingly, another study has demonstrated that microRNA 548 (miRNA) regulates the expression of HMGB1 in fetal membrane tissues with very minimal expression in preterm chorioamniotic fetal membranes (Son et al., 2019). Induced expression of miRNA 548 in isolated epithelial cells from fetal membranes decreased HMGB1 levels and pro-inflammatory cytokine levels. Together these data strongly suggest that although HMGB1 secretion is increased during the cellular stress of infection it may also have a role in a sterile immune response working in autocrine and paracrine ways that could contribute to PTB.

A possible role for serum amyloid A1 (SAA1) in ECM remodeling was assessed in amnion fetal membrane explants. Human amnion fetal membrane explants treated with SAA1 showed increased protein expression of MMP-1, MMP-8, and MMP-13 compared to control treatment (Wang et al., 2019). The increased expression of MMPs suggest a potential role for SAA1 in ECM remodeling in fetal membranes after rupture.

TLR9 recognizes unmethylated CpG-containing DNA sequences in addition to bacterial and viral DNA to activate an immune response, similarly to all the other DAMPs. cffDNA is one of the few nucleic acid DAMPs that have been studied in the context of a placental source of cffDNA. It is thought that cffDNA released from the placenta can circulate in the maternal plasma to activate TLR9 on leukocytes and macrophages. This then can induce a pro-inflammatory immune response that triggers labor, which ultimately leads to birth (Phillippe, 2014). A study in support of the importance of this group of DAMPs, demonstrated that TLR9 is expressed on fetal membranes from term placentas in the presence and absence of labor (Beck et al., 2019). Indeed, its levels are highly expressed in AEC (Gillaux et al., 2011; Sato et al., 2016) and AMC (Sato et al., 2016). Exosomes from senescent AECs are known to package cffDNA and HMGB1 that could also to signal within the fetal membranes acting as a trigger for parturition (Sheller-Miller et al., 2017). In further support of this, human peripheral blood mononuclear cells (PBMCs) treated with fetal DNA or CpG DNA increased IL-6 secretion and pregnant Bagg Albino (BALB/c) mice injected with fetal DNA, CpG DNA or LPS increased the number of resorbed fetuses. In addition, TLR9 knockout mice treated with fetal DNA showed decreased numbers of resorbed mice, whereas those treated with the TLR9 inhibitor Chloroquine reduced fetal resorptions and IL-6 production. This suggests a role for TLR9 in promoting PTB upon cffDNA treatment (Scharfe-Nugent et al., 2012). More studies are needed to show the mechanism by which cffDNA could contribute to the activation of parturition and also what role it has in the fetal membranes where the expression of its detection receptors is high in AEC and AMC (Sato et al., 2016).

To our knowledge only one study has specifically tested the activation of AMC by DAMPs. In this study the authors elegantly show that the TLR4 receptor is present and able to be activated by the DAMP, fetal fibronectin, on AMC (Haruta et al., 2013) resulting in the increase in the activity of MMP-1 and MMP-9.

It is clear that due to the high incidence of infection driven PTB, including those with pPROM, the role of TLR and their ligands in the fetal membranes with and without chorioamnionitis has been the subject of some study. To date, only a few papers have produced data linking DAMPs (as opposed to PAMPs) and the fetal membranes. These have mostly focused on the production of HMGB1, which has been confirmed to increase in amniotic fluid of human fetal membranes with pPROM (Romero et al., 2011), and is induced by intrauterine infection with LPS, in sheep (Regan et al., 2016). Even though much of the current work that is focused on DAMPs in the fetal membranes is AEC centric, the data provides strong evidence

that DAMP production by AMC would produce inflammation, perhaps in an autocrine fashion, and this would likely influence the integrity of the amnion. In addition, DAMPs are produced by many of the other cells important for the maintenance of pregnancy (Phillippe, 2014; Nadeau-Vallee et al., 2016). They could also activate AMC in a paracrine fashion and contribute to the increase in inflammation and MMPs, which could ultimately lead to membrane rupture.

DAMP/PAMP PRR PATHWAY MANIPULATION: POTENTIAL THERAPEUTIC STRATEGIES FOR FETAL MEMBRANE MAINTENANCE

There are a multitude of developing therapies based on PRRs recognition of their ligands for the resolution of diseases with an inflammatory signature. Indeed, in other disease and disorder fields like sepsis for example, DAMP manipulation is being tested by the antagonism of HMGB1 (Yang et al., 2005). Although this body of work is expansive, it will not be focused on here as it would warrant its own focused review article. Here we aim to describe the studies that have been specifically focused on the manipulation of this pathway in the tissues of pregnancy, including the fetal membranes where possible, and therefore show promise for future development of strategies to address the maintenance of the integrity of this tissue (**Figure 2**).

Signaling events activated by the DAMP and PAMP ligands are numerous, and complex, with an ever-expanding repertoire of players. Some of these molecules overlap between pathways, while others are more specific. This broad network provides numerous potential targets for manipulation and his has been explored due to their potential for other causes of PTB, but rarely for their direct effects on the fetal membranes. This is because it is highly likely that DAMPs and PAMPs working through their PRRs also have a role in myometrial activation and cervical ripening. This body of work has provided some exciting data (**Figure 2**) that may be used to help us discern the ability for some of the already tested strategies, or develop novel ones, that could be used to help manipulate the timing of membrane rupture too.

It has been suggested that anti-TLR monoclonal antibodies could be good therapeutic target to decrease inflammation driven adverse perinatal outcomes (Wong et al., 2018) and thus it should be considered whether they may also serve as good PTB therapies. Indeed, neutralizing antibodies against TLR4 reduce the percentages of decidual invariant natural killer cells, decreasing inflammation (Li et al., 2015). Soluble TLRs have also been suggested as potential markers or treatment sites for those conditions where they are understood to have a key role (Abrahams et al., 2013). However, it still remains to see their effects elsewhere in the other tissues when pregnant. An alternative strategy in the same vein has been to use soluble RAGE as a therapeutic tool to decrease inflammation. Pretreatment with these inhibited LPS-induced preterm uterine contractility, cytokines, and prostaglandins in Rhesus monkeys (Waldorf et al., 2008), supporting the premise that this approach has merit for further exploration in pregnancy.

An alternate approach designed to target pathways more specifically has been to knockdown downstream signaling for the PRRs. Interferon regulatory factor (IRF) 5 knockdown leads to less inflammation in myometrium (Lim et al., 2018) and thus inhibitors for IRF1 have also been suggested as a therapeutic strategy due to their direct role in TLR signaling (Lim et al., 2016). Other studies testing the potential of TLR4 inhibitors have been successful at decreasing levels of inflammation associated with its activation. Indeed, levels of Tollip, an inhibitor of TLR4 that is known to correlate with PE severity (Nizyaeva et al., 2019) and the TLR4 antagonist (+)-naloxone (Chin et al., 2016) were shown to suppress cytokine expression. Although these studies were performed in the placenta and myometrium/decidua, respectively, they provide promising data for developing this approach in fetal membranes.

Other work has focused specifically on PAMP stimulated TLR response manipulation. Thus, several different biomolecules have been shown to inhibit inflammation or PTB in animal models due to their ability to decrease TLR ligand induced responses. Surfactant-A intrauterine injection was found to significantly decrease TLR ligand induced inflammation and inhibit preterm delivery via TLR2 (Agrawal et al., 2014). As GSK3 α and β activity is increased in fetal membranes after labor, the GSK3β inhibitor CHIR99021 was tested and found to decrease LPS stimulated pro-inflammatory cytokines, TNFα, IL-1β, IL-6, IL-8, prostaglandins and MMP-9 in myometrial cells and fetal membranes cells (Lim and Lappas, 2015). In addition, the protein Pellino 1 was shown to regulate TLR and TNF signaling, by decreasing TLR2/6, TLR3, TLR5 and the pro-inflammatory cytokines IL-8, IL-1β, and IL-6 (Lim et al., 2018). When the addition of Tim3 was tested, it was found to protect decidual cells from TLR extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) dependent apoptosis and inflammation (Wang et al., 2015).

Decreases in TLR signaling by the removal of their key partners or decreasing receptor numbers have also been studied. The elimination of Mincle, a sensor for lipids, was shown to lead to decreased effect of TLR ligands to cause inflammation in uterus (Lim and Lappas, 2019). In addition, GIT2 knockdown, in myometrial and amnion cells also lead to decreased inflammation in response to TLR ligands (Lim and Lappas, 2019). The P2X7 receptor, which has been shown to regulate IL-1β release from gestational tissues working in concert with TLRs has also been studied. The inflammation it produced on stimulation with a specific receptor agonist, was inhibited by the progesterone, prolactin and an NF-κB inhibitor (Maneta et al., 2015). Finally, treatment with 15-deoxy-delta-12, 14-prostaglandin J2 (15d-PGJ2) affected TLR4 by blocking its NF-κB induced inflammation, reducing preterm labor in a mouse model (Pirianov et al., 2009).

It remains to be seen how the further development of these potential therapeutic targets will continue. With such a large number of signaling pathways, receptors and ligands involved, there are many other targets that are yet to be investigated. However, which of the manipulated mechanisms will have too many unwanted effects, or that have an unwanted

global biological effect on other organs and the fetus, remains to be determined. Regardless, interest in these pathways is gathering momentum due to their potential to increase our understanding of the mechanisms at play and to prevent the rupture of the membranes. Due to their central role in infection and non-infection driven inflammation, they also have enormous potential for treatments for the other etiologies of PTB (Ekman-Ordeberg and Dubicke, 2012).

SUMMARY

The trigger that initiates tissue remodeling in preparation for parturition remains elusive. This missing stimulus includes that which activates the weakening cascade of the fetal membranes in preparation for its rupture. Understanding this is not only important to determine what happens in normal pregnancy but also because the timing of this event is crucial for healthy pregnancy outcomes. If rupture does not occur in a timely fashion it can result in pROM or pPROM leaving the fetus vulnerable to infection and distress. It can also precipitate PTB, as pPROM is evident in approximately one third of all cases of premature delivery. It is currently thought that the change that may switch on the process of membrane weakening is an increase in cellular stress. This is already known to increase toward the end of gestation in the form of physical distension of the fetal membrane tissue and also the build-up of ROS. It is well established that DAMPs are generated by many forms of cell stress and their general biological function is to raise the alarm within tissues. They do this by producing a number of pro-inflammatory cytokines, MMPs and often lead to apoptosis. They accomplish this through their interaction with numerous PRRs and their downstream signaling pathways, often achieving their influence on inflammation by activating the transcription factor NF-κB. Much of what we understand about the role of DAMPs, especially in the fetal membranes, comes from work in other tissue types or from consideration of data from PAMP ligand action. This is because these ligands also work through many of the same PRRs. However, these data should be viewed with caution; PAMPs and DAMPs have been seen to result in differential signaling through the same PRR. Despite this, numerous similarities that have been described in their actions. PAMPs are well established to cause infection driven membrane rupture, and so it is tempting to postulate that the DAMP counterparts are also able to do this. These molecules could therefore be the link between the biophysical and biochemical changes that happen toward term to weaken the fetal membranes. This idea has been least studied from the perspective of understanding the ECM DAMPs. Perhaps this is because they are one of the more difficult molecules to study in this tissue, given that most researchers are working on it at in term state when much of the ECM has already started to breakdown. Thus, we need to build new models to study the interaction and generation of ECM DAMPs with the AMC within this layer of the amnion. There have been several studies that have focused on understanding the potential of the manipulation of the DAMP, PAMP, PRR system, with an eye to future therapies for various PTB etiologies. Although still in their infancy, it is tempting to speculate that more work focused in this vein will indeed not only solidify the key role of these molecules in parturition, but provide much needed prevention and identification biomolecular targets to help us address the high rates of PTB in the United States.

AUTHOR CONTRIBUTIONS

JP contributed to all the written sections focused on the different receptors and also drew and annotated **Figure 1**. CS contributed to the PAMPs and DAMPs written sections and assisted with editing. CK-W contributed to all sections, tables, and figures.

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MINI REVIEW
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Functional Genomics of Healthy and Pathological Fetal Membranes

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Premature preterm rupture of membranes (PPROM), rupture of fetal membranes before 37 weeks of gestation, is the leading identifiable cause of spontaneous preterm births. Often there is no obvious cause that is identified in a patient who presents with PPROM. Identifying the upstream molecular events that lead to fetal membrane weakening presents potentially actionable mechanisms which could lead to the identification of at-risk patients and to the development of new therapeutic interventions. Functional genomic studies have transformed understanding of the role of gene regulation in diverse cells and tissues involved health and disease. Here, we review the results of those studies in the context of fetal membranes. We will highlight relevant results from major coordinated functional genomics efforts and from targeted studies focused on individual cell or tissue models. Studies comparing gene expression and DNA methylation between healthy and pathological fetal membranes have found differential regulation between labor and quiescent tissue as well as in preterm births, preeclampsia, and recurrent pregnancy loss. Whole genome and exome sequencing studies have identified common and rare fetal variants associated with preterm births. However, few fetal membrane tissue studies have modeled the response to stimuli relevant to pregnancy. Fetal membranes are readily adaptable to cell culture and relevant cellular phenotypes are readily observable. For these reasons, this is now an unrealized opportunity for genomic studies isolating the effect of cell signaling cascades and mapping the fetal membrane responses that lead to PPROM and other pregnancy complications.

Keywords: genomics, fetal membranes, transcriptomics, preterm birth, gene regulation and expression

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INTRODUCTION

Preterm birth remains a major public health challenge affecting 10% of pregnancies in the United States (World Health Organization, 2016). The leading identifiable cause of preterm birth is premature preterm rupture of membranes (PPROM) (Mercer, 2010). Preeclampsia is characterized as shallow trophoblast invasion leading to incomplete spiral artery remodeling. It affects 5% of pregnancies and is an iatrogenic cause of prematurity and the leading cause of maternal and perinatal death (Souza et al., 2013).

These adverse pregnancy outcomes all have multifactorial causes incorporating genetic and environmental risk factors.

Functional genomics assays aim to define the relationships between the human genome and epigenome; the environment; and molecular, cellular, and organismal phenotypes. The past decade has been transformative for functional genomics, owing largely to high-throughput short read sequencing providing quantitative and genome-wide readout for many functional genomic assays. Such assays are particularly adept at identifying differential activity that may result from changes in the environment, such as hormone exposures or immune insults [e.g., (McDowell et al., 2018; Pulido-Salgado et al., 2018)]. Today, there are a vast array of genome-wide functional genomic technologies available to measure a wide variety of aspects of gene expression, DNA methylation, histone positioning and modifications, transcription factor binding, gene regulatory activity, other factors that indicate gene regulation (Arnold et al., 2013; Mundade et al., 2014; Finotello and Di Camillo, 2015; Tirado-Magallanes et al., 2016). Through those studies, there is now extensive information about the gene regulatory state of diverse cells and tissues.

For the purposes of this review, we define functional genomic assays as those that scan large fractions of the genome for evidence of regulatory activity. In the context of human disease studies, such regions are a promising starting point for subsequent efforts to discover causative biological mechanisms. Follow-up is then needed to evaluate the biological consequences of identified regulatory regions, both in terms of the effects on cellular and organismal phenotypes and also in terms of the effects of non-coding genetic variation on their activity.

Functional genomics studies have primarily focused on immortalized cell models, ostensibly because they are highly proliferative and robust. However, recent advances in the adaptation of functional genomics protocols for use on limited primary cells and tissues have created the potential to study more physiologically relevant cell models [e.g., (Vento-Tormo et al., 2018; Chung et al., 2019)]. In the context of preterm birth, fetal membranes are a key tissue of interest, and ideally suited for genomic analysis due to their availability and amenability to cell culture. Protocols to culture and expand amnion and chorion cells were developed in the 1980s (Burgos and Faulk, 1981). Culturing primary cells in these systems allows for interrogation of fetal membranes by genomic assays. In addition, several genomic assays are now feasible from a limited number of primary cells, and even single cells, making culturing unnecessary (Jia et al., 2018; Wang et al., 2019). Together these developments have led to a number of genomic assays comparing fetal membrane tissues from healthy pregnancies to those involved in preeclampsia, early pregnancy loss and preterm birth.

DEFINING FETAL MEMBRANE SPECIFIC REGULATORY STATE

Functional genomic assays on fetal membrane samples have been completed both by large genomics consortia such as ENCODE and Roadmap Epigenomics (The Encode Project Consortium, 2012; Roadmap Epigenomics Consortium et al., 2015), as well as by individual labs (Kim et al., 2012; Lim et al., 2012; **Table 1**). The consortia efforts have focused on amnion and chorion tissues from full term and second trimester samples. Across those samples, they have measured genome-wide gene expression using RNA-seq, cytosine methylation using whole genome bisulfite sequencing, and the locations of covalent histone modifications, indicators of active gene regulation, using ChIP-seq.

RNA-seq typically measures mRNA transcript levels that can be compared among tissue types to identify tissue specific transcription (Mortazavi et al., 2008). Bisulfite sequencing assays identify methylated cytosine that are typically thought to be related to silencing of gene activation. They do so by using sodium bisulfite treatment to convert unmethylated cytosines to uracil prior to PCR amplification and sequencing (Meissner et al., 2008). When sequencing treated and untreated DNA, the uracil bases sequence as thymine in treated samples but remain as cytosine in untreated samples (Clark et al., 1994). Finally, ChIP-seq assays use antibodies to isolate DNA-bound proteins including histones (Johnson et al., 2007; Robertson et al., 2007). ChIP-seq can detect histone subunits altered with post translational modifications that influence DNA affinity and, in turn, how accessible the DNA is to transcriptional machinery (Zhou et al., 2011). Together, these datasets can establish a baseline of gene regulatory state across among membranes from healthy pregnancies, and an assessment of the changes in gene regulation between the second and third trimester.

Additional studies have investigated the gene expression (Kim et al., 2012) of healthy term placental tissue types including fetal membranes. Transcriptomic analysis shows that placental cell types are more similar to each other when compared to other adult tissue types, but each placental cell type shows a subset of tissue type specific gene expression as well (Kim et al., 2012). The epithelial specific splice regulator ESPR1 is significantly unregulated in fetal membrane tissue, particularly the amnion, above other tissue types. In the amnion, the relative expression of ESPR1 is 50% higher than that of next highest tissue of the 16 adult tissues measured. Substantial alternative splicing and novel isoforms specific to the fetal membranes have been found by RNA-seq studied of healthy term membranes (Kim et al., 2012).

To further define healthy gene regulation in fetal membranes, microarray-based gene expression studies compared activated amnion from late term non-laboring elective Cesarean-sections, defining activation as high NF-κB protein levels similar to the levels observed in post-delivery samples (Lim et al., 2012). That activation of the amnion is an early step that stimulates the synthesis of prostaglandins, cytokines and chemokines initiating the beginning of labor. Although all the samples were non-laboring, some samples were closer to the onset of labor at the time of C-section and could be differentiated from more quiescent samples. Activation of the amnion is associated with an up regulation of a cell death and cancer associated gene network, consistent with an increase in apoptosis in activated amnion (Lim et al., 2012). An additional gene network associated with cell-to-cell signaling is also unregulated in response to activation, consistent with the role of the amnion as an early initiator of labor induction.

TABLE 1 | Published genomic analyses in fetal membrane tissues.

Source	Tissue Type	Disease State	Assay
Roadmap Epigenomics	Amnion, basal plate, chorion smooth, trophoblast, placental villi	Healthy full term and 2nd trimester c-sections	mRNA-seq, histone modification ChIP-seq
ENCODE	Amnion, basal plate, chorion, trophoblast, placental villi	Healthy full term and 2nd trimester c-sections	mRNA-seq, microRNA-seq, DNase-seq, histone modification ChIP-seq
Tromp et al., 2004	Chorioamnion	Preterm Labor, PPROM, Term in labor and term not in labor	Microarray
Montenegro et al., 2009	Chorioamnion	Term in labor and not laboring and preterm labor	microRNA microarray
Nhan-Chang et al., 2010	Amnion and Chorion	Healthy full term spontaneous rupture of membranes	Microarray
Li et al., 2011	Amnion mesenchymal cells	Healthy term c-section stimulated with IL-1B	Microarray
Kim et al., 2012	Amnion, chorion	Healthy full term c-section	RNA-seq
Kim et al., 2013	Amnion	Term in labor and not laboring and preterm labor	Illumina Methylation BeadChip
Lim et al., 2012	Amnion	Healthy full term c-section	Microarray
Kim et al., 2016	Amnion epithelial	Healthy and preeclamptic term c-section	mRNA-seq
Söber et al., 2016	Chorionic villi	Recurrent pregnancy loss and elective abortion 2nd trimester	mRNA-seq, MicoRNA-seq
Wang et al., 2017	Chorionic villi	Recurrent miscarriages and elective abortion	lncRNA microarray
Jiang et al., 2018	Chorionic trophoblasts	Healthy term c-section stimulated with LPS	RNA-seq, Whole Genome Bisulfite Sequencing
Pereyra et al., 2019	Amnion and Chorion	Severe preterm and full term spontaneous labor	RNA-seq
Yang et al., 2019	Chorionic villi	Early embryonic arrest and elective abortion 2nd trimester	mRNA-seq, MicoRNA-seq

TABLE 2 | Published genetic analyses of preterm birth.

Source	Disease State	Assay
McGinnis et al., 2017	Offspring of preeclamptic pregnancies and controls	SNP array
Modi et al., 2017	Healthy term and PPROM African American infants	Whole Exome sequencing
Modi et al., 2018	Healthy term and PPROM African American infants	Whole Exome sequencing
Liu et al., 2019	Varied gestation duration	SNP array
Tiensuu et al., 2019	Term and spontaneous preterm birth	SNP array

HEALTHY VERSUS PATHOLOGICAL FETAL MEMBRANES

Functional genomic studies are particularly powerful for identifying molecular differences between different cell states, such as between fetal membrane tissues from healthy pregnancies and those with pregnancy complications. Differentially regulated genes from these studies can identify molecular pathways that may be either causal or a downstream consequence, and in the best cases can nominate new therapeutic targets. In fetal membrane tissues, such comparative studies have been done to compare healthy tissues to those from patients with preterm birth, recurrent early pregnancy loss, and preeclampsia.

Preterm Birth and Membrane Rupture

Due to the integral role fetal membranes play in maintaining pregnancy or stimulating parturition, a substantial amount of research has been devoted to understanding changes in gene regulation that occur during the onset of parturition. RNA-seq analyses have revealed hundreds of gene expression changes that occur between the site of membrane rupture and distal membrane sites in term spontaneously ruptured membranes. For example, Nhan-Chang et al. (2010) used microarrays to identify 677 differentially expressed genes at the site of rupture compared to a distal site in the chorion (Nhan-Chang et al., 2010). The differentially expressed genes were enriched for increased expression of genes involved in complement and coagulation at the site of rupture, suggesting a role for immune activation in membrane integrity. Genes related to extracellular matrix-receptor interaction were most altered at the site of rupture, consistent with the role of the extracellular matrix in maintaining fetal membrane integrity (Bryant-Greenwood, 1998).

Because signaling cascades leading to fetal membrane rupture can be informative in identifying the causes of membrane rupture, directly comparing the gene expressed at the site of rupture in preterm and term deliveries can give more direct insight into the cause of PPROM. The gene expression patterns of term membrane samples are more internally consistent whereas preterm samples are more variable (Pereyra et al., 2019). The variability in preterm samles suggests multiple signalling cascades lead to preterm birth, distinct from those leading to term births. Despite this variability, 270 significantly differentially expressed genes with a >2-fold gene expression change were found when comparing membranes from early preterm births to membranes from term births. Several genes from the tumor necrosis factor (TNF), chemokine and voltage gated potassium channel families were significantly differentially regulated. Inflammatory and immunological pathways were also significantly up-regulated in preterm birth, consistent with a role for immune responses in the etiology of some preterm birth (Velez et al., 2008).

Functional genomic studies can also identify genes of interest for specific causes of preterm birth. In one example, comparisons of gene expression in fetal membranes between preterm labor with intact membranes and membranes from PPROM patients identified Proteinase Inhibitor 3 (PI3) having

significantly decreased expression in PPROM samples (Tromp et al., 2004). Immunohistochemical staining confirmed the decreased expression levels of PI3 protein expression in fetal membranes collected from patients presenting with PPROM (Tromp et al., 2004). PI3 is an anti-proteinase that may protect the extracellular matrix from degradation by proteases, specifically Elastase 2 and Proteinase 3 (Guyot et al., 2005). In other cell types, TNFα and IL1β have been found to induce PI3 production (Pfundt et al., 2000; Bingle et al., 2001). PI3 was not previously implicated in preterm birth or PPROM specifically, but genome wide studies suggest decreased expression, due to genetic or environmental signaling, could lead to PPROM.

Functional genomic studies have also identified differential epigenetic states which may contribute to or result from such gene expression differences. Differential DNA methylation in amnion between term and preterm pregnancies in labor and term pregnancies not in labor show the majority of changes in methylation occur at the onset of labor. A large portion of the differentially methylated genes are associated with non-coding RNA and imprinted genes (Kim et al., 2013). Of the regions that show changes in methylation between preterm and term, enrichment in genes related to cation transport, cytokine production and extracellular matrix receptor interactions were observed, supporting differential expression studies demonstrating similar patterns in functional enrichment (Kim et al., 2013).

MicroRNAs, another layer of control, regulate gene expression post-transcriptionally through binding to and destabilizing mRNA molecules (Ambros, 2004). Although most miRNA lack experimentally validated targets, computational predictions can suggest genes that may be involved in biological processes (Ekimler and Sahin, 2014). Ten miRNA were specifically differentially regulated between term in labor and preterm labor membranes, all of which were down regulated (Montenegro et al., 2009). Additionally, the RNA processor Dicer was down regulated suggesting miRNAs play a key role in the parturition process at term but not preterm. Coupled with gene expression data, these studies can show regulation that occurs at the onset of labor that separates preterm and term processes.

Early Pregnancy Loss

Early embryonic arrest affects approximately 10% of pregnancies with rates increasing as the age of couples trying to conceive increases (Larsen et al., 2013). Pregnancy loss is due to factors including uterine abnormalities, abnormal chromosomes and infection pathologies but genetic factors can also lead to a pregnancy loss (Xu et al., 2016). Transcriptomic profiles from chorionic villi of early embryonic arrest samples compared to gestation age matched elective termination samples show differentially expression in PI3K-Akt signaling pathway, Jak-STAT pathway and complement and coagulation signaling cascades (Yang et al., 2019). One study looking specifically at long non-coding RNA that are differentially regulated in chorionic villi between patients with recurrent miscarriage and those undergoing an elective abortion found up regulation of steroid hormone biosynthesis and extracellular matrix interaction and well as down regulation of TGF-beta signaling and apoptosis pathways (Wang et al., 2017). An additional study comparing chorionic villi from recurrent pregnancy loss couples, defined as having five or more miscarriages, to elective termination samples shows a substantial down regulation of key small non-coding RNA as well as histone genes (Sõber et al., 2016). Those results suggest that chorionic villi cells begin repressing key cellular processes leading to loss of the pregnancy.

Preeclampsia

Preeclampsia is a common disease that is the leading cause of pregnancy associated mortality and morbidity for both the mother and child (Roberts and Cooper, 2001). Shallow trophoblast invasion and impaired remodeling of the uterine spiral arteries are associated with preeclampsia (Pennington et al., 2012). Gene expression of amnion epithelial cells from healthy and preeclamptic c-sections were compared to understand the underlying disease etiology. Functional annotation of differentially expressed genes identified pathways involved in extracellular matrix-receptor interaction and focal adhesion. Additional validation studies showed differential expression of matrix metalloproteinases that control degradation of the extracellular matrix (Kim et al., 2016).

RESPONSE TO STIMULI

Understanding signaling events that cause membrane rupture can suggest specific pathways misregulated in PPROM. Testing specific response pathways can connect early signaling events from *in vitro* stimulus response studies to *in vivo* studies that examine the progression of labor. Such *in vitro* studies can circumvent the limitation that observational studies are necessarily correlative and thus cannot differentiate between the cause and consequence. Additionally, *in vitro* stimulus-response studies can identify intermediate steps leading to the onset of phenotype that observational studies miss due to strict limits on tissue collection during pregnancy. For example, *in vitro* functional genomic studies of fetal membranes cells responding to inflammatory stimuli can reveal the direct effects of those signals on pathways related to cell proliferation, adhesion, or apoptosis that may impact the timing of membrane rupture. Indeed, studies of cultured amnion mesenchyme cells exposed to an IL1β challenge for up to 8 h showed transcriptional dynamics reflecting an immediate immune challenge compared to sustained response. The early responsive genes showed signatures of NF-κB activity, a well-documented effector of IL-1 signaling (Cogswell et al., 1994; Greten et al., 2007; Liu et al., 2017). Later responsive genes had more diverse transcription factor binding sites indicative of a cascade of downstream gene regulatory events. Those secondary factors including the AP-1 family transcription factors that were not regulated by the initial IL-1β response (Li et al., 2011). Similarly, immune challenges to chorionic trophoblast cells through lipopolysaccharide (LPS) show an increase in gene expression related to cytokine production and response, although this signaling appears to be mediated through the STAT1-STAT3 pathway

(Jiang et al., 2018). While differential DNA methylation is detected following LPS stimulation, 2 h of LPS induction may not be enough to detect significant changes in methylation. Together, these studies demonstrate the types of insights possible from functional genomic studies of fetal membrane cells after *in vitro* exposures. However, many of the common signals in pregnancy such as hormonal changes, oxidative stress and mechanical force changes remain to be investigated.

GENETIC STUDIES OF FETAL MEMBRANES

Transcriptomic and DNA methylation studies can take on additional informative power when combined with genetic association studies. Most variants identified in genome wide association studies are found in non-coding regions (Zhang and Lupski, 2015). Integration with functional genomic data sets can reveal candidate causal mechanisms, including target genes of clinical importance (Lowe and Reddy, 2015). The primary challenge is that the lead signal in a genetic association study is in linkage disequilibrium with many surrounding variants. Thus, the patterns of linkage disequilibrium in the study population limit resolution, often to >10 kb. Functional genomic datasets can suggest which variants in that LD-based region are most likely to have regulatory activity (Conde et al., 2013). That approach was used to identify a variant that abolishes a transcription factor binding site that represses interleukin 1 family members in fetal membranes (Liu et al., 2019). The variant identified was suggested to have a gene expression effect on multiple members of the interleukin 1 family including IL1A, IL36G, and IL36RN. A similar approach was also used in a genome wide association study of early preterm and term infants. Several significant variants near the gene SLIT2 were identified that overlaps regions of DNase hypersensitivity, suggesting regulatory activity, in several fetal tissues including the amnion (Tiensuu et al., 2019).

The combination of epigenomic data and genome wide association studies has also been employed for other pregnancy complications affecting the fetal membranes, including preeclampsia. A genome wide association study that incorporated both maternal and fetal DNA variants identified a variant near the gene FLT1 from the offspring of pregnancies associated with preeclampsia (McGinnis et al., 2017). The evidence for the effect of this variant was built by the fact that Roadmap Epigenomics incorporating many different epigenetic datasets, such as histone modifications and open chromatin sites, labeled this site as a putative enhancer in both amnion and trophoblast cell types.

While many genome wide association studies detect common non-coding variants from large populations, rare coding variants can also contribute to disease. In these cases, whole exome sequencing is often employed to detect these variants. A whole exome sequencing study of PPROM cases and healthy term controls in an African American population identified 10 rare variants more common in PPROM cases than term controls in native regulators of innate immunity, LPS detoxifying enzymes and antimicrobial protein genes (Modi et al., 2017). An additional follow up replication study replicated two of the variants in the genes DEFB1 and MBL2, both thought to be antimicrobial proteins in fetal membranes (Modi et al., 2018). The use of genomic sequencing technologies can detect both common and rare variants associated with fetal membranes pathologies. Studies identifying variants relevant to these pathologies are outlined in **Table 2**.

FUTURE STUDIES

While the number of studies comparing regulation between healthy and pathological membranes is growing, the data available remains sparse. Published studies have largely focused on comparing transcriptomic data or DNA methylation between cases and controls, often using microarray measurements that are noisier and have less dynamic range than sequencing-based methods (Zhao et al., 2014). In addition, few studies on fetal membranes have deposited raw data in publicly available databases, limiting benefit to other fetal membrane researchers. All together missing are assays of chromatin accessibility or histone modification in fetal membrane tissue type which can add more information about different levels of regulation and suggest transcription factors responsible for signaling that leads to pregnancy complications. Expanding studies of the response to relevant stimuli in fetal membrane tissues is a major opportunity. Studies thus far have focused on cellular responses to inflammatory stimuli but further studies looking at mechanical stress, hormone signaling and oxidative stress using *in vitro* tissue models in addition to cellular models to replicate the structural complexity of fetal membranes and cellular interaction can help add to a more complete understanding of the signaling that leads to PPROM, preterm birth, preeclampsia or early pregnancy loss.

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Study of sRAGE, HMGB1, AGE, and S100A8/A9 Concentrations in Plasma and in Serum-Extracted Extracellular Vesicles of Pregnant Women With Preterm Premature Rupture of Membranes

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Preterm premature rupture of membranes (PPROM), defined as rupture of fetal membranes prior to 37 weeks of gestation, complicates approximately 2–4% of pregnancies and is responsible for 40% of all spontaneous preterm births. PPROM arises from complex pathophysiological pathways with a key actor: inflammation. Sterile inflammation is a feature of senescence-associated fetal membrane maturity. During specific steps of sterile inflammation, cells also release highly inflammatory damage-associated molecular pattern markers (DAMPs), such as high-mobility group box 1 (HMGB1) or S100A8/A9, known to link and activate the receptor for advanced glycation end products (RAGE). The objective of this study was to measure longitudinally during pregnancy concentrations of the soluble form of RAGE (sRAGE) and its main ligands (AGE, HMGB1, S100A8/A9) in blood specimens. We studied 246 pregnant women (82 with PPROM and 164 matched control pregnant women without complications) from a cohort of 7,866 pregnant women recruited in the first trimester and followed during pregnancy until delivery. sRAGE, AGE, HMGB1, and S100A8/A9 concentrations were measured in plasma and in serum-extracted extracellular vesicles from first trimester (T1), second trimester (T2), and delivery (D). In plasma, we observed, in both PPROM and control groups, (i) a significant increase of HMGB1 concentrations between T1 vs. T2, T1 vs. D, but not between T2 vs. D; (ii) a significant decrease of sRAGE concentrations between T1 and T2 and a significant increase between T2 and D; (iii) a significant decrease of AGE from T1 to D; (iv) no significant variation of S100A8/A9 between trimesters. In intergroup comparisons (PPROM vs. control group), there were no significant differences in time variation taking into account the matching effects.

There was a correlation between plasma and serum-extracted extracellular vesicle concentrations of sRAGE, AGE, HMGB1, and S100A8/A9. Our results suggest that the rupture of fetal membranes (physiological or premature) is accompanied by a variation in plasma concentrations of sRAGE, HMGB1, and AGE. The study of RAGE and its main ligands in extracellular vesicles did not give additional insight into the pathophysiological process conducting to PPROM.

Keywords: preterm premature rupture of membranes, extracellular vesicles, soluble receptor for advanced glycation end products, advanced glycation end products, high-mobility group box 1, S100A8/A9

INTRODUCTION

Preterm premature rupture of membranes (PPROM), defined as rupture of fetal membranes prior to 37 weeks of gestation, complicates approximately 2–4% of all pregnancies and is responsible for 40–50% of all preterm births (Naeye and Peters, 1980; Mercer et al., 2000). PPROM arises from complex, multifaceted pathophysiological pathways where the inflammation axis plays a major role (Menon and Richardson, 2017). Indeed, recent reports indicated that PPROM may be associated with sterile inflammation in the fetal membranes (Romero et al., 2015). In support of this hypothesis, it has been shown that histological chorioamnionitis in the presence of a negative amniotic fluid culture increases the risk of preterm birth (Park et al., 2017). Sterile inflammation is a feature of senescence-associated fetal membranes maturity and is characterized mostly by the presence of inflammatory biomarkers, growth factors, and matrix degrading enzymes (Coppe et al., 2008). During the specific steps of sterile inflammation, senescent, stressed, or necrotic cells release highly inflammatory damage-associated molecular pattern markers (DAMPs) (Menon and Richardson, 2017). High-mobility group box 1 (HMGB1) is one of the DAMPs that have been linked to parturition (Sheller-Miller et al., 2017; D'Angelo et al., 2018). In a mouse model, intra-amniotic administration of HMGB1 induces spontaneous preterm labor and birth (Gomez-Lopez et al., 2016). Moreover, it was observed that HMGB1 induces an inflammatory response, partially mediated by the inflammasome, in the fetal membranes (Plazyo et al., 2016). This intra-amniotic inflammasome activation was highlighted *in vivo* in human (Gomez-Lopez et al., 2019). HMGB1 is a known ligand of receptor for advanced glycation end products (RAGE), and the RAGE system is associated with pregnancy complications as preeclampsia or PPROM (Naruse et al., 2012; Rzepka et al., 2015). Moreover, AGEs could be implicated in PPROM with blood levels significantly higher in pregnant women complicated with PPROM (Kansu-Celik et al., 2019). Calprotectin (or S100A8/A9) is also a known ligand of RAGE (Pruenster et al., 2016) implicated in some pregnancy pathologies as preeclampsia (Pergialiotis et al., 2016).

Extracellular vesicles are a heterogeneous group of cell-derived membranous structures comprising exosomes (50–150 nm) and microvesicles (50–500 nm up to 1 μm), which originate from the endosomal system or which are shed from the plasma membrane, respectively (van Niel et al., 2018). The study of maternal plasma exosomes determines pathways associated with PPROM including non-specific inflammation or oxidative stress (Menon et al., 2019). The RAGE system (receptor and ligands) has not been specifically studied in maternal blood exosomes. However, some DAMPs, such as HMGB1 have been identified as present in oxidative-stressed amnion epithelial cell-derived exosomes (Sheller-Miller et al., 2017). Furthermore, *in vitro*, amnion epithelial cell exosomes lead to an increased inflammatory response in maternal uterine cells, suggesting that fetal cell exosomes may act as a signal to parturition in choriodecidua and migrate into the maternal circulation (Hadley et al., 2018). Combining maternal characteristics and environmental and clinical known risk factors (Bouvier et al., 2019) to candidate biomarkers may in the future result in proposing a clinically predictive model identifying asymptomatic women at higher risk of PPROM.

In this context, taking advantage of a large cohort of pregnant women recruited prospectively at the beginning of pregnancy, we investigated the changes in the concentrations of the soluble form of RAGE (sRAGE) and its main ligands (AGE, HMGB1, S100A8/A9) in plasma and in the serum-extracted extracellular vesicles from first trimester to delivery to better understand the potential role of the RAGE system in PPROM.

MATERIALS AND METHODS

Study Design and Participants

This is a case/control study of sRAGE, HMGB1, AGE, and S100A8/A9 concentrations in plasma samples and serum-extracted exosomes of pregnant women with PPROM from an already constituted prospective biobank for which blood samples were collected [research program funded by the CIHR Institute of Human Development, Child and Youth Health Initiative (Grant Number: NRFHPG-)]. The biobank includes samples from 7,866 pregnant women recruited at the CHU de Québec-Université Laval between April 2005 and March 2010 and followed during pregnancy until delivery (Forest et al., 2014). Participants gave their informed written consent, and the study was approved by the Ethics Committee of the CHU de Québec [initial approval date: November 9, 2004, project 5-04-10-01 [95.05.17], SC12-01-159]. Cases were selected from all pregnant women with PPROM for whom three successive blood samples were collected and then frozen: one during the first trimester (T1), one during the second trimester (T2), and one at delivery (D). In the control group, we selected pregnant women (two control for one case) with delivery

at term (after 37 weeks of gestation) and for whom three plasma and serum samples were collected and then frozen (T1, T2, and D). Women in the control group were matched with those in the case group on the following criteria: maternal age (±5 years), gestational age at T1 sample (±1 week), gestational age at T2 sample (±3 weeks), storage time at -80°C (±6 months). A total of 246 pregnant women (82 with PPROM and 164 matched control pregnant women) were selected.

Serum Extracellular Vesicle Extraction

For total extracellular vesicles from 30 to 120 nm isolation from 738 serum samples (three samples T1, T2, and D for 246 women), we used a kit (ref 4478360) from Invitrogen™ (Carlsbad, California, United States) using 450 µl of serum and following the manufacturer's instructions. Then, for extraction of total proteins from extracellular vesicles, we used Invitrogen™ kit (ref 4478545) following the manufacturer's instructions. The assay of total proteins in the extracellular vesicle extracts was carried out using a Vista® analyzer (Siemens, Munich, Germany). The assay of apolipoprotein B (Apo B) in 16 extracellular vesicle extracts (eight from the control group and eight from the PPROM group) was carried out using a Vista® analyzer (Siemens, Munich, Germany).

ELISA of Soluble Receptor for Advanced Glycation End Products, High-Mobility Group Box 1, Advanced Glycation End Products, and S100A8/A9

The concentrations of sRAGE, HMGB1, AGE, and S100A8/A9 in 738 plasma samples (three samples T1, T2, and D for 246 women) and in 738 serum-extracted extracellular vesicle samples (three samples T1, T2, and D for 246 women) was measured by the ELISA method using MyBioSource® kits (San Diego, California, United States) following the manufacturer's instructions (ref MBS2515963, MBS024146, MBS2000151, and MBS7606803, respectively). The concentrations of sRAGE, HMGB1, AGE, and S100A8/A9 of each serum extracellular vesicle sample were normalized against total protein concentrations.

Statistics

Statistical analyses were performed using Stata software, Version 13 (StataCorp, College Station, Texas, United States). All tests were two-sided, with a Type I error set at 0.05. Continuous data were expressed as mean and standard deviation (SD) or median and interquartile range (IQR) according to statistical distribution. The assumption of normality was assessed by using the Shapiro–Wilk test. The comparisons between the PPROM and control groups, for non-repeated data, were performed using Student *t*-test or Mann–Whitney test when the assumptions of *t*-test were not met for continuous parameters. Chi-square test or, if applicable, Fisher's exact test were applied for categorical variables. The relation between continuous variables (AGEs, sRAGE, HMGB1, S100A8/A9 concentrations) in serum-extracted extracellular vesicles and in plasma was analyzed estimating correlation coefficients, Pearson or Spearman according to the statistical distribution and applying a Sidak's type I error correction to take into account multiple comparisons. These correlations' results were illustrated with a color-coded heat map.

Random-effects models for repeated data were performed to compare the evolution of AGEs, sRAGE, HMGB1, and S100A8/A9 plasma concentrations and serum-extracted extracellular vesicle concentrations between groups (PPROM and controls). The following fixed effects were measured: time (T1, T2, D), group and *time* × *group* interaction, taking into account between- and within-participant variability (subject as random-effect). The normality of residuals from these models was studied using the Shapiro–Wilk test. When appropriate, a logarithmic transformation was proposed to achieve the normality of dependent outcome. A Sidak's type I error correction was applied to perform multiple comparisons.

RESULTS

Description of the Cohort

Of the 7,866 pregnant women recruited for the biobank, 189 women presented a PPROM (2.4%). Of these, 82 fulfilled the criteria of disposing of three blood samples. Therefore, a total of 246 pregnant women (82 with PPROM and 164 matched control pregnant women, 1:2 ratio) were selected. No significant differences ($p = 0.7$) were observed between the mean age of mothers in the control group (29.5 years, SD: 4.1) and the PPROM group (29.3 years, SD: 4.2) (**Table 1**). Some risk factors of PPROM were found significantly higher in the PPROM group as nulliparity, past history of PPROM, gestational diabetes mellitus, smoking during pregnancy (**Table 1**). A significant difference ($p < 0.001$) for the gestational age at delivery was expectedly observed between the PPROM group [36 weeks, interquartile range (IQR): 35.1–36.4] and the control group (38.7 weeks, IQR: 38.1–39.3) (**Table 1**).

Assays in Plasma

A significant decrease of median concentrations of AGEs in both PPROM and control groups was observed between T1 and T2, T2 and D, and T1 and D (see p^1 and p^2 in **Table 2** for the PPROM and control group, respectively; **Figure 1A**). These variations in

TABLE 1 | Characteristics of the case (PPROM) and control groups.

		PPROM group	Control group	p
n		82	164	/
Mean age of mothers (SD) in years		29.3 (4.2)	29.5 (4.1)	0.7
Nulliparity in%		58.5	43.3	0.02
Past history of PPROM in%		15.6	0	<0.001
Gestational diabetes mellitus in%		14.6	6.7	0.04
Smokers during pregnancy in%		21.3	11.7	0.04
Median	First trimester	15.1 (14–16.6)	15 (14–15.6)	0.37
gestational age	Second trimester	27.9 (26.1–28.5)	27.9 (26.2–28.4)	0.97
(IQR) in weeks	Delivery	36 (35.1–36.4)	38.7 (38.1–39.3)	<0.001

IQR, interquartile range; PPROM, preterm premature rupture of membranes; SD, standard deviation.

TABLE 2 | Median AGEs, sRAGE, HMGB1, and S100A8/A9 plasma concentrations from 82 women in the PPROM group and 164 women in the control group at three points: first trimester (T1), second trimester (T2), and delivery (D).

	PPROM group (*n* = 82)		Control group (*n* = 164)		p^3	p^4
	Median (IQR)	p^1 T1 vs. T2 T2 vs. D D vs. T1	Median (IQR)	p^2 T1 vs. T2 T2 vs. D D vs. T1		
AGEs (ng/ml)						
First trimester	3,569 (2,665–4,532)	0.04	3,651 (2,951–4,345)	<0.001	0.16	0.41
Second trimester	3,313 (2,676–4,232)	<0.001	3,353 (2,692–4,125)	<0.001	0.17	0.76
Delivery	2,885 (2,284–3,818)	<0.001	2,993 (2,439–3,694)	<0.001	0.97	0.43
sRAGE (pg/ml)						
First trimester	1,819 (915–3,234)	0.001	1,873 (1,170–3,402)	<0.001	0.33	0.55
Second trimester	1,556 (808–2,625)	<0.001	1,719 (971–2,670)	<0.001	0.65	0.30
Delivery	2,178 (1,092–3,721)	0.06	2,418 (1,432–3,665)	<0.001	0.15	0.21
HMGB1 (ng/ml)						
First trimester	22.3 (14.1–33.9)	<0.001	19.1 (13–31.3)	<0.001	0.65	0.32
Second trimester	30.4 (22.6–44.3)	0.24	30.4 (21.5–40.7)	0.09	0.86	0.29
Delivery	27.8 (20.5–37.7)	<0.001	27.7 (19–38.9)	<0.001	0.78	0.26
S100A8/A9 (ng/ml)						
First trimester	332 (157–865)	0.77	344 (168–692)	0.61	0.98	0.68
Second trimester	344 (216–644)	0.14	325 (204-678)	0.55	0.33	0.64
Delivery	297 (163–568)	0.24	316 (157–673)	0.93	0.31	0.23

p^1 and p^2: intragroup comparison between T1, T2, and D in the PPROM group (p^1) and in the control group (p^2) (random-effects models for repeated measures). p^3: intergroup comparison: interaction time × group, taking into account the matching effect (random-effects models for repeated measures). p^4: comparison (of medians) between the PPROM group and the control group, taking into account the matching effect. AGEs, advanced glycation end products; HMGB1, high-mobility group box 1; IQR, interquartile range; PPROM, preterm premature rupture of membranes; sRAGE, soluble receptor of advanced glycation end products.

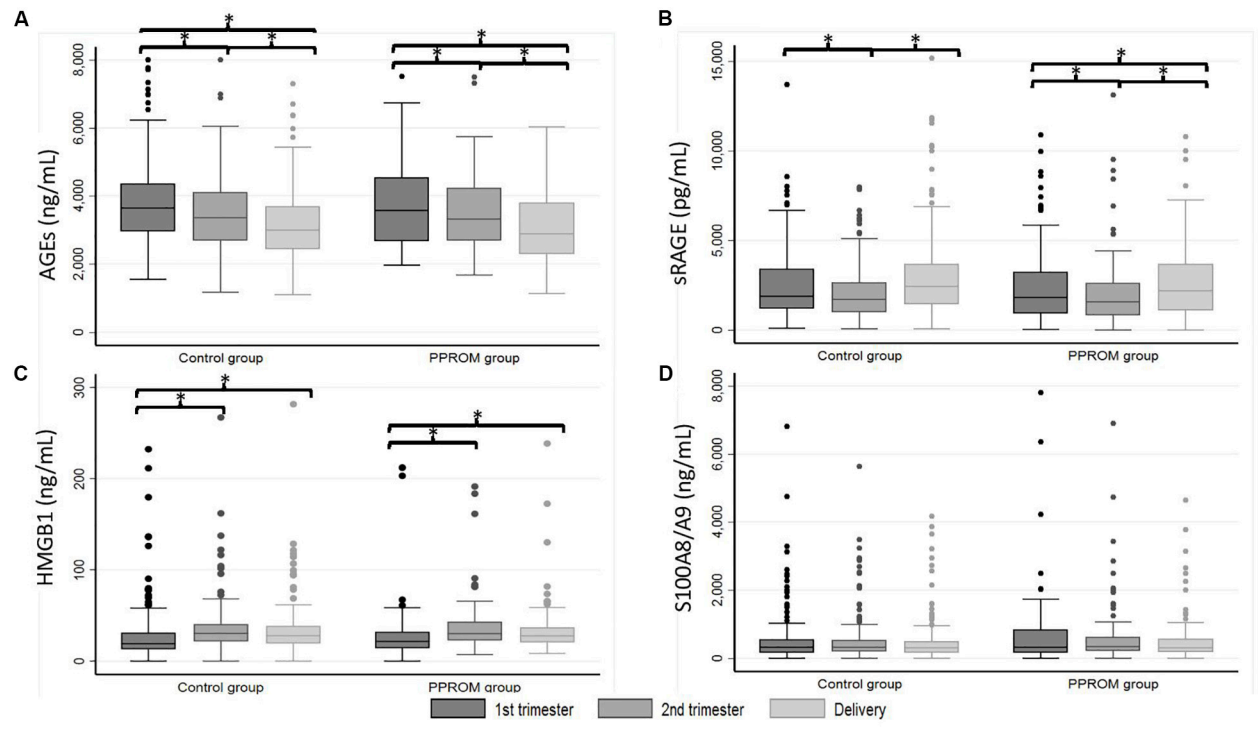

FIGURE 1 | Box plot of AGEs **(A)**, sRAGE **(B)**, HMGB1 **(C)**, and S100A8/A9 **(D)** plasma median concentrations from 82 women in the PPROM group and 164 women in the control group at three points: first trimester, second trimester, and delivery. *$p < 0.05$ (random-effects for repeated measures). AGEs, advanced glycation end products; HMGB1, high-mobility group box 1; PPROM, preterm premature rupture of membranes; sRAGE, soluble receptor of advanced glycation end products.

concentrations observed during pregnancy were not significantly different between the PPROM and control groups (see p^3 in **Table 2**). Similarly, for each sampling time (T1, T2, D), the medians of plasma concentrations are not significantly different between the PPROM and control groups (see p^4 in **Table 2**).

For sRAGE in both PPROM and control groups, a significant decrease of median concentration between T1 and T2 and then a significant increase between T2 and D were observed (see p^1 and p^2 in **Table 2** for the PPROM and control group, respectively; **Figure 1B**). At delivery, the sRAGE concentration was significantly higher than at T1 in the control group ($p < 0.001$) but not in PPROM ($p = 0.06$). These variations in concentrations observed during pregnancy were not significantly different between the PPROM and control groups (see p^3 in **Table 2**). Similarly, for each sampling time (T1, T2, D), the medians of plasma concentrations were not significantly different between the PPROM and control groups (see p^4 in **Table 2**).

For HMGB1 in both PPROM and control groups, a significant increase of median concentration was observed between T1 and T2 (and also T1 and D) followed by a stagnation between T2 and D (see p^1 and p^2 in **Table 2** for the PPROM and control groups, respectively; **Figure 1C**). These variations in concentrations observed during pregnancy were not significantly different between the PPROM and control groups (see p^3 in **Table 2**). Similarly, for each sampling time (T1, T2, D), the medians of plasma concentrations were not significantly different between the PPROM and control groups (see p^4 in **Table 2**).

For S100A8/A9, in both PPROM and control groups, no significant variations of median concentrations were observed between T1, T2, and D (see p^1 and p^2 in **Table 2** for the PPROM and control groups, respectively, see p^3 in **Table 2** and **Figure 1D**). Similarly, for each sampling time (T1, T2, D), the medians of plasma concentrations were not significantly different between the PPROM and control groups (see p^4 in **Table 2**).

Assays in Serum-Extracted Extracellular Vesicles

Similar results as those obtained in plasma are presented in **Table 3** were observed. The variations of concentration during pregnancy were similar as in plasma (see p^1 and p^2 in **Table 3** for the PPROM and control groups, respectively; **Figure 2**). For all four markers (AGEs, sRAGE, HMGB1, and S100A8/A9) measured in serum-extracted extracellular vesicles, the variations in concentration observed during pregnancy were not significantly different between the PPROM group and the control group (see p^3 in **Table 3**). Similarly, for each sampling time (T1, T2, D), the medians of serum-extracted extracellular vesicles concentrations were not significantly different between the two groups (see p^4 in **Table 3**). Moreover, AGEs, sRAGE, HMGB1, and S100A8/A9 concentrations were significantly correlated between the plasma and the serum-extracted extracellular vesicles for both the PPROM group (expect for AGEs at delivery) and the control group (**Figure 3**).

The assay of Apo B in 16 extracellular vesicles extracts (eight from the control group and eight from the PPROM group) was carried out. The results were found to be below or at the lower limit of linearity (<0.26 g/L).

TABLE 3 | Median AGEs, sRAGE, HMGB1, and S100A8/A9 serum-extracted extracellular vesicle concentrations from 82 women in the PPROM group and 164 women in the control group at three points: first trimester (T1), second trimester (T2), and delivery (D).

	PPROM group (n = 82)		Control group (n = 164)		p^3	p^4
	Median (IQR)	p^1 T1 vs. T2 T2 vs. D D vs. T1	Median (IQR)	p^2 T1 vs. T2 T2 vs. D D vs. T1		
AGEs (μ g/g of protein)						
First trimester	97.4 (64.7–117.5)	0.01	90 (66.3–115.1)	0.20	0.53	0.40
Second trimester	83.3 (61.2–107)	0.77	86.3 (63–110.3)	0.97	0.85	0.89
Delivery	81.4 (59.4–105.2)	0.01	81.6 (60.1–101.6)	0.22	0.41	0.91
sRAGE (ng/g of protein)						
First trimester	43.3 (30.6–57.3)	0.004	40.6 (30.3–57.6)	< 0.001	0.42	0.92
Second trimester	36.7 (29.6–48.8)	0.07	34.7 (27.9–48.4)	0.06	0.73	0.43
Delivery	40.1 (32.3–54.2)	0.29	37.9 (27.4–51.7)	0.001	0.25	0.27
HMGB1 (μ g/g of protein)						
First trimester	3.4 (1.8–4.7)	< 0.001	2.8 (1.8–4.6)	0.001	0.23	0.59
Second trimester	4.1 (2.4–6.2)	< 0.001	3.5 (2.1–5.2)	< 0.001	0.21	0.11
Delivery	3.2 (1.8–4.4)	0.63	2.9 (1.7–4.7)	0.64	0.95	0.66
S100A8/A9 (μ g/g of protein)						
First trimester	125 (93–174)	0.008	123 (86–186)	0.84	0.11	0.80
Second trimester	143 (107–226)	0.001	140 (95–205)	0.25	0.11	0.16
Delivery	127 (91–183)	0.39	119 (85–191)	0.34	0.99	0.89

p^1 and p^2: intragroup comparison between T1, T2, and D in the PPROM group (p^1) and in the control group (p^2). p^3: intergroup comparison: interaction time × group, taking into account the matching effect (random-effects models for repeated measures). p^4: comparison (of medians) between the PPROM group and the control group, taking into account the matching effect (random-effects models for repeated measures). AGEs, advanced glycation end products; HMGB1, high-mobility group box 1; IQR, interquartile range; PPROM, preterm premature rupture of membranes; sRAGE, soluble receptor of advanced glycation end products.

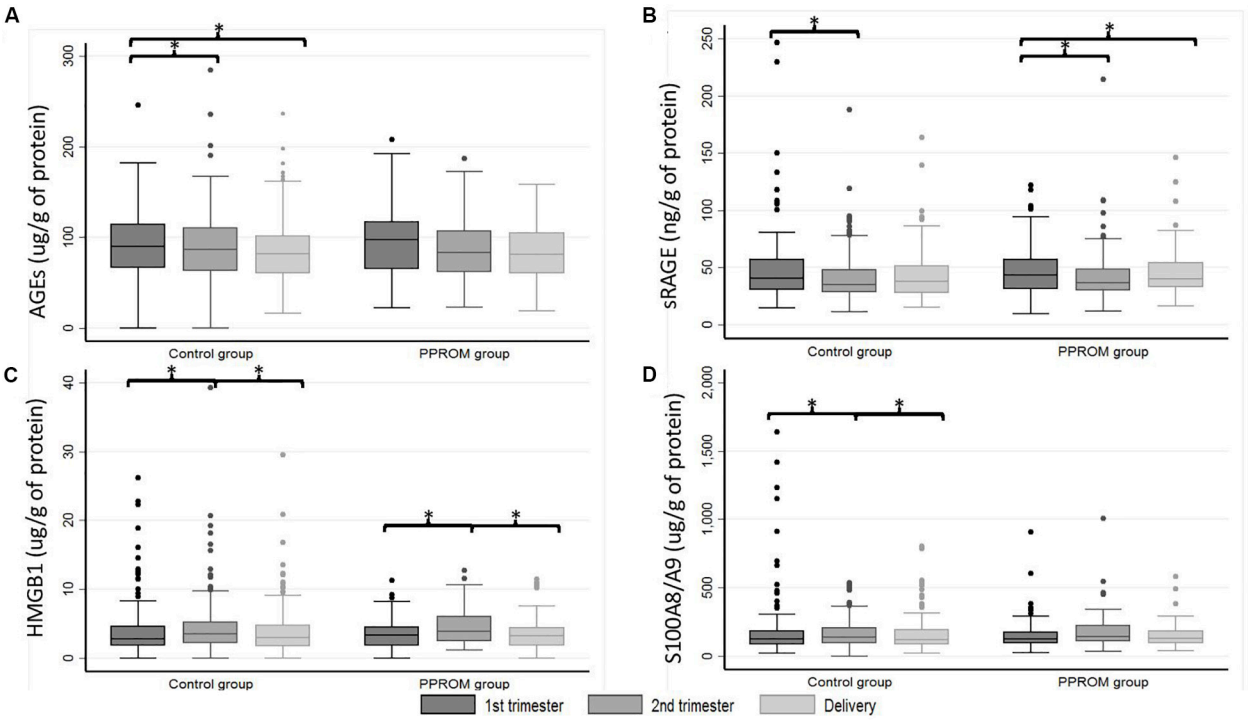

FIGURE 2 | Box plot of AGEs **(A)**, sRAGE **(B)**, HMGB1 **(C)**, and S100A8/A9 **(D)** serum-extracted extracellular vesicle median concentrations from 82 women in the PPROM group and 164 women in the control group at three points: first trimester, second trimester, and delivery. *$p < 0.05$ (random-effects for repeated measures). AGEs, advanced glycation end products; HMGB1, high-mobility group box 1; PPROM, preterm premature rupture of membranes; sRAGE, soluble receptor of advanced glycation end products.

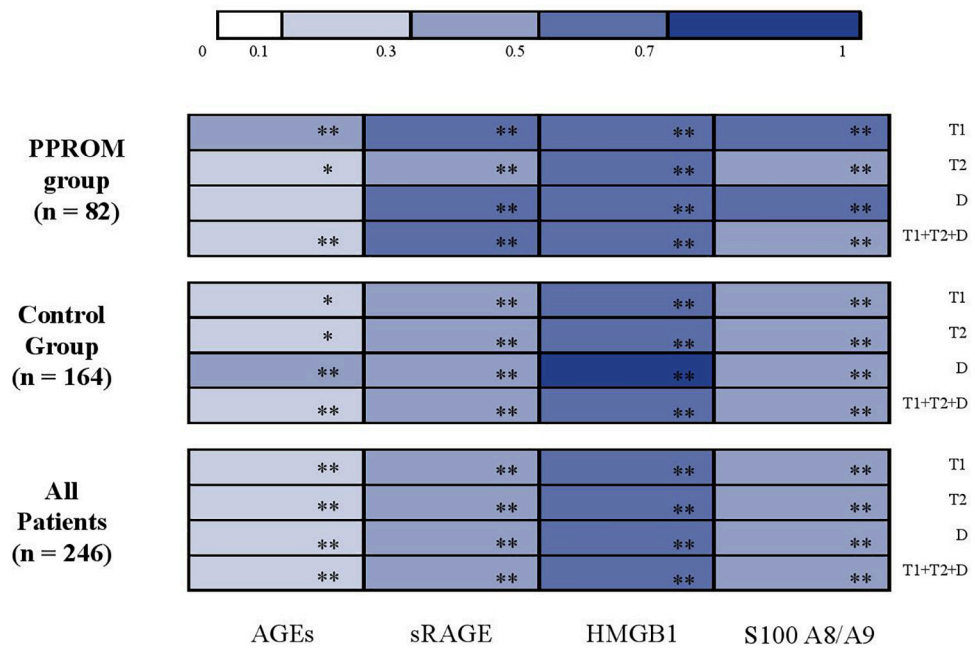

FIGURE 3 | Correlation analysis between AGEs, sRAGE, HMGB1, and S100A8/A9 concentrations in serum-extracted extracellular vesicles and in plasma (color-coded heat map plot). AGEs, advanced glycation end products; D, delivery; HMGB1, high-mobility group box 1; PPROM, preterm premature rupture of membranes; sRAGE, soluble receptor of advanced glycation end products; T1, first trimester; T2, second trimester. *$p < 0.05$; **$p < 0.001$.

DISCUSSION

We studied for the first time, on a large number of pregnant women (with and without PPROM), the kinetics from the first trimester to delivery of the plasma concentrations (and serum-extracted exosomes) of four major actors of the RAGE system: sRAGE and three of its ligands, HMGB1, AGEs, and S100A8/9. Among the 189 women with PPROM (2.4% of the cohort), 82 had three blood samples during their pregnancies and were matched to normal controls in a ratio of 1–2. Despite the potential bias linked to the matching criteria, we identified the clinical known risk factors for PPROM as described in a previous study (Bouvier et al., 2019).

There were no significant differences in the serum concentration of total circulating extracellular vesicles between pregnant women with and without PPROM. However, it was proposed that the study of maternal plasma-extracted exosomes could determine pathways associated with PPROM including non-specific inflammation or oxidative stress (Menon et al., 2019). The specific study of biomarkers of the RAGE system in serum-extracted extracellular vesicles of pregnant women was relevant to identify an earlier signal of suffering from the fetal membranes. In this study, we observed that this is not the case and that the concentrations are strongly correlated to those of "total serum," for the studied actors of RAGE signaling, in contrast to the cellular networks in the work of Menon's team. Flow cytometry could have been used to determine whether RAGE or studied DAMPs are present on the surface of or inside the extracellular vesicles, but this was not available in our current setting. Another limitation of the study is the absence of characterization of the extracellular vesicles extracted from the serum (van Niel et al., 2018) and of the study of the cellular or tissue origin of these vesicles. Measurement of Apo B below or at the lower limit of linearity (<0.26 g/L) confirmed the absence of contamination of the preparations of extracellular vesicles.

So far, studies on blood levels of sRAGE as PPROM or prematurity risk factor show discordant results (Hajek et al., 2008; Germanova et al., 2010; Bastek et al., 2012; Rzepka et al., 2016). In our study, no differences in plasma sRAGE concentrations were observed between the control and PPROM groups, as already described (Hajek et al., 2008; Rzepka et al., 2016). Indeed, Rzepka et al. (2016) found the same results and found the endogenous secretory RAGE (esRAGE) more interesting as a potential biomarker of PPROM. Bastek et al. (2012) found lower serum concentrations of sRAGE in women who gave birth prematurely including spontaneous premature labor with intact membranes and thus confirmed results of a smaller study (Germanova et al., 2010). Conversely, Hajek et al. (2008) found, in a pilot study, higher values of serum concentrations of sRAGE in women who gave birth prematurely. In our study, an interesting kinetics is observed with a decrease of sRAGE plasma concentration between T1 and T2, and then an increase between T2 and D. Germanova et al. (2010) described exactly the opposite but on a smaller cohort of 79 women with only 25 measurements per trimester. These different conclusions could be due to the presence of different size of cohorts, type of circulating RAGE, and various assays used to measure sRAGE.

Concerning the RAGE ligands in plasma, we observed no differences in S100A8/19 concentrations between the PPROM group and the control group and no variations during pregnancy. Also, we found no differences in plasma AGE concentrations between the PPROM and control groups. These results are in contradiction with those of a recent study on a small cohort of 46 pregnant women (nine with PPROM and 37 without PPROM) where the blood concentration of AGEs at T1 was higher in women with PPROM (Kansu-Celik et al., 2019). Noteworthy, a decrease in the serum concentration of AGEs during pregnancy was also observed in a previous study where the kinetics were studied between the second trimester and delivery (Quintanilla-Garcia et al., 2018). In our study, no differences in plasma HMGB1 concentrations were observed between the PPROM and control groups. However, variations in plasma HMGB1 concentrations during pregnancy were observed with a significant increase between T1 and T2 and then stagnation between T2 and D in both PPROM and control groups. It was previously observed that senescent fetal membranes contribute to sterile inflammation by generation of DAMPs, like HMGB1 (Menon et al., 2016). Also, an increase in the expression of HMGB1, with activation of the RAGE pathway, in the placenta of women with PPROM has been demonstrated (Yan et al., 2018). Our results show the same kinetics in women with PPROM and in control women. HMGB1 has a physiological implication and can be at the "frontiers in physiology." Based on our blood results, the mechanistic link between the increases in plasma concentrations of HMGB1 and sRAGE and the decrease in the plasma concentration of AGEs should be further investigated. It has been reported that the sRAGE–AGE complex becomes degraded in the spleen or liver (Ramasamy et al., 2008).

CONCLUSION

In conclusion, our results suggest that the rupture of fetal membranes (physiological or premature) may be related to RAGE activation possibly by the ligand HMGB1. If a more important production of HMGB1 occurring during pregnancy with PPROM could not be detected directly by a higher concentration of HGMB1 in PPROM vs. control group, an indirect proof by the different kinetics of sRAGE between first trimester and delivery could be proposed.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the CHU de Québec. The

patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DB analyzed and interpreted the data and wrote the initial version of the manuscript. J-CF and YG were in charge of the research program on pregnancy complications, designed the study, and assisted with the interpretation of the data and writing of the manuscript. NB and EB supervised the trial and data collection. DB and NB carried out serum exosome extractions and all assays. BP provided statistical advice for the study design and analyzed the data. VS, LB, and DG experts in PPROM, reviewed the manuscript, assisted with the interpretation of the data. All authors substantially contributed to its revision.

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In vivo Assessment of Supra-Cervical Fetal Membrane by MRI 3D CISS: A Preliminary Study

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In approximately 8% of term births and 33% of pre-term births, the fetal membrane (FM) ruptures before delivery. *In vitro* studies of FMs after delivery have suggested the series of events leading to rupture, but no *in vivo* studies have confirmed this model. In this study, we used a three-dimensional constructive interference in steady state (3D-CISS) sequence to examine the FM at the cervical internal os zone during pregnancy; 18 pregnant women with one to three longitudinal MRI scans were included in this study. In 14 women, the FM appeared normal and completely intact. In four women, we noted several FM abnormalities including cervical funneling, chorioamniotic separation, and chorion rupture. Our data support the *in vitro* model that the FM ruptures according to a sequence starting with the stretch of chorion and amnion, then the separation of amnion from chorion, next the rupture of chorion, and finally the rupture of amnion ruptures. These findings hold great promise to help to develop an *in vivo* magnetic resonance imaging marker that improves examination of the FMs.

Keywords: amnion, chorion, fetal membrane, preterm birth, premature rupture of membranes, preterm premature rupture of membranes, magnetic resonance imaging

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INTRODUCTION

During pregnancy, the fetus is surrounded by amniotic fluid contained within a fetal membrane (FM). FM is composed of the amnion, which faces the fetus, and the chorion, which contacts the maternal decidua. In a healthy pregnancy, the FM is critical for maintaining a pregnancy until delivery (Parry and Strauss, 1998; Menon and Richardson, 2017). However, in about 8% of pregnancies, the FM ruptures before labor, which is called premature rupture of membranes (PROM). FM rupture before 37 weeks of gestation, termed preterm prelabor rupture of membranes (PPROM), is responsible for approximately one-third of preterm births and is the most common identifiable factor associated with preterm birth (Mathews and MacDorman, 2010; Waters and Mercer, 2011; Martin et al., 2012). Currently, there is no easy way to predict PPROM in early pregnancy, and thus the prevention is very limited.

To solve this problem, we first need to understand the mechanisms of FM rupture. Several investigators have attempted to do so by performing *in vitro* mechanical test on FM after delivery

(Artal et al., 1976; Lavery and Miller, 1979; Helmig et al., 1993; Oyen et al., 2004). For example, data from Arikat et al. and Strohol et al. suggest that FM rupture follows this sequence: (1) Amnion and chorion stretch together under load; (2) amnion separates from chorion; (3) chorion ruptures; (4) amnion distends further, non-elastically; and (5) amnion ruptures (Arikat et al., 2006; Strohl et al., 2010). Ultrasound, an imaging modality widely used clinically to monitor pregnancy *in vivo*, can detect some signs associated with PROM and PPROM, such as FM thickness (Frigo et al., 1998; Severi et al., 2008; Başaran et al., 2014; Nunes et al., 2016) and chorioamniotic separation (Devlieger et al., 2003). The FM region that appears to be most prone to rupture is near the internal cervical os (McLaren et al., 1999). However, this para-cervical weak zone is often difficult to visualize by transvaginal ultrasound because of the low contrast between the FM and the maternal decidua (Severi et al., 2008). Strong *in vivo* evidence is still absent in the literature.

Here, we proposed to visualize the FM near the internal cervical os using magnetic resonance (MR) images acquired with a sequence named three-dimensional constructive interference in steady state (3D-CISS). This sequence provides both high spatial resolution and excellent contrast between the cerebrospinal fluid (high signal from water) and tissue structures (lower signal). And thus it is commonly used in clinical procedures to evaluate fine structures, such as cranial nerves surrounded by cerebrospinal fluid (Yoshino et al., 2003; Yousry et al., 2005). In MR images, the difference of signal intensity between amniotic fluid (high signal) and the FM (low/intermediate signal) is similar to the difference of signal intensity between cerebrospinal fluid and nerves, and the FM has similar thickness as nerves. Therefore, the 3D-CISS sequence is able to visualize the FM near the internal cervical os. In our study, we performed 3D-CISS MR imaging on 18 women at one to three time points between 20 and 36 weeks of gestation. And we report the result of four women who had evidence of abnormal FM structure. Our data suggest that the *in vivo* FM rupture sequence matches what proposed from *in vitro* studies (Arikat et al., 2006; Strohl et al., 2010).

MATERIALS AND METHODS

Participants

This study was approved by the Washington University in St. Louis Institutional Review Board (protocols 201612140, 201707152). Participants were recruited by research nurses from the patient population attending the Obstetrics and Gynecology Clinic and the Women's Health Center in the Barnes-Jewish Hospital Center for Outpatient Health. Participants were included if they were 18 years of age or older and had a healthy singleton pregnancy. Participants were excluded if they had a twin pregnancy or a contraindication to MRI. Before imaging, all patients were screened for MRI safety and provided written informed consent. Age, body mass index, and other clinical information were recorded for all participants. Pregnancy outcomes were collected from the medical records. Term birth was defined as birth between 37 0/7 weeks of gestation and 42 0/7 weeks of gestation (Goldenberg et al., 2008). Preterm birth was defined as birth between 20 0/7 weeks of gestation and 36 6/7 weeks of gestation (Goldenberg et al., 2008). PROM was defined as rupture of membranes before labor. PPROM was defined as rupture of membranes followed by labor before 37 weeks of gestation (Simhan and Canavan, 2005; Goldenberg et al., 2008).

MRI Acquisition

Every patient underwent MRI examination one, two, or three times between 20 and 36 weeks of gestation. A Siemens Magnetom Vida 3T whole body MRI scanner and a 30-channel phased-array torso coil (Erlangen, Germany) were used to acquire a series of sagittal view T2 weighted images (T2WI), with a half-Fourier acquisition single-shot turbo spin echo sequence and the following parameters: repetition time, 1800 ms; echo time, 94 ms; matrix, 320 × 650; flip angle, 140°; layer thickness, 4.0 mm; slice spacing, 0.8 mm; number of layers, 25. For the 3D-CISS sequence, parameters were as follows: repetition time, 7.71 ms; echo time, 3.70 ms; flip angle, 50°; acquisition number, 1; acquisition matrix, 640 × 640; field of view, 300 mm × 300 mm; bandwidth, 460 Hz per pixel; slice thickness, 1 mm; and in-plane resolution, 0.33 mm × 0.33 mm. The total acquisition time for both T2WI and 3D-CISS was 7 min.

Image Analysis

Magnetic resonance images were independently analyzed by two radiologists (WQ and WW, with 10-year and 1-year of experience, respectively, in analyzing abdominal MR images) who were blinded to pregnancy outcomes. A consensus was reached in cases of discordance. The following imaging characteristics were evaluated: cervical funneling, chorioamniotic separation, and chorion or amnion rupture.

RESULTS

Between April 2019 and February 2020, 18 pregnant women were recruited for this study. Their mean age was 33.5 ± 12.1 years, and their mean body mass index at first prenatal visit was 23.8 ± 5.3 kg/m^2. Demographic and clinical details of the 18 women included in this study are presented in **Table 1**. A total of 43 MRI scans were performed on these 18 patients.

Fourteen patients had normal-appearing FM in which the amnion, chorion, and decidua were intact and indistinguishable from one another at all imaging time points. For example, in the patient images shown in **Figures 1A–C**, the FM was completely intact at 20, 32, and 36 weeks' gestation, though we noted some suspended FM material in the cervical canal at all three time points. None of the 14 patients with normal, intact FM had PPROM or PROM, and all 14 delivered at term.

Four patients had both cervical funneling, in which the FM protruded into the cervix, and chorioamniotic separation, in which amniotic fluid was visible between the amnion and chorion, detectable in at least one of their MRI scans.

In patient #1, the FM appeared normal at 20 weeks (**Figure 1D**). However, at 32 weeks, this patient had cervical funneling with amniotic fluid and FM protruding into the cervix

TABLE 1 | Demographic and clinical characteristics of pregnant women.

	Total (*n* = 18)	ROM at labor (*n* = 17)	PPROM (*n* = 1)
Age, years, median (range)	26.5 (19–35)	26 (19–35)	25
Body mass index, kg/m^2, average (range)	27.68 (18.5–39.0)	27.66 (18.5–39.0)	28.0
Race/ethnicity, *n* (%)			
African American	16 (88.9)	15 (88.2)	1 (100)
Caucasian	2 (11.1)	2 (11.8)	0
Asian	0	0	0
Other	0	0	0
Multiparous, *n* (%)	16 (88.9)	15 (88.2)	1 (100)
Nulliparity	2 (11.1)	2 (11.8)	0

(**Figure 1E**). At 36 weeks, amniotic fluid was visible between amnion and chorion, indicating chorioamniotic separation (**Figure 1F**). This patient did not have PPROM or PROM and delivered at term.

In patient #2, the FM showed cervical funneling and partial chorioamniotic separation at 32 weeks and complete chorioamniotic separation at 36 weeks (**Figures 2A,B**). This patient did not have PPROM or PROM and delivered at term.

In patient #3, the FM showed cervical funneling and partial chorioamniotic separation at 24 weeks and complete chorioamniotic separation at 32 and 36 weeks (**Figures 2C–E**). This patient did not have PPROM or PROM and delivered at term.

In patient #4, the FM showed deeper cervical funneling, chorioamniotic separation, and chorionic rupture at 36 weeks (**Figure 3**). This patient developed PPROM 6 h after the MRI scan and delivered preterm (36 2/7 weeks).

DISCUSSION

In our study, the longitudinal 3D-CISS MRI data provide the first *in vivo* evidence to support the first three steps of the model proposed by Arikat et al. regarding the sequence of events leading to FM rupture and PROM or PPROM. In the first step of their model, the FM stretches and protrudes into the cervix when the cervical internal os dilates to cause cervical funneling. This is evident in patient #1 at 32 weeks. In step 2, the amnion partially or completely separates from the chorion, as is evident in patient #1 at 36 weeks, patient #2 at 32 and 36 weeks, patient #3 at 24, 28, and 32 weeks, and patient #4 at 36 weeks. In step 3, further cervical internal os dilation leads to additional FM stretch and chorion rupture as seen in patient #4 at 36 weeks. In step 4, the amnion distends further. Finally, in step 5, the amnion ruptures, leading to PPROM or PROM. We present a schematic of the first three steps of this model in **Figure 4**.

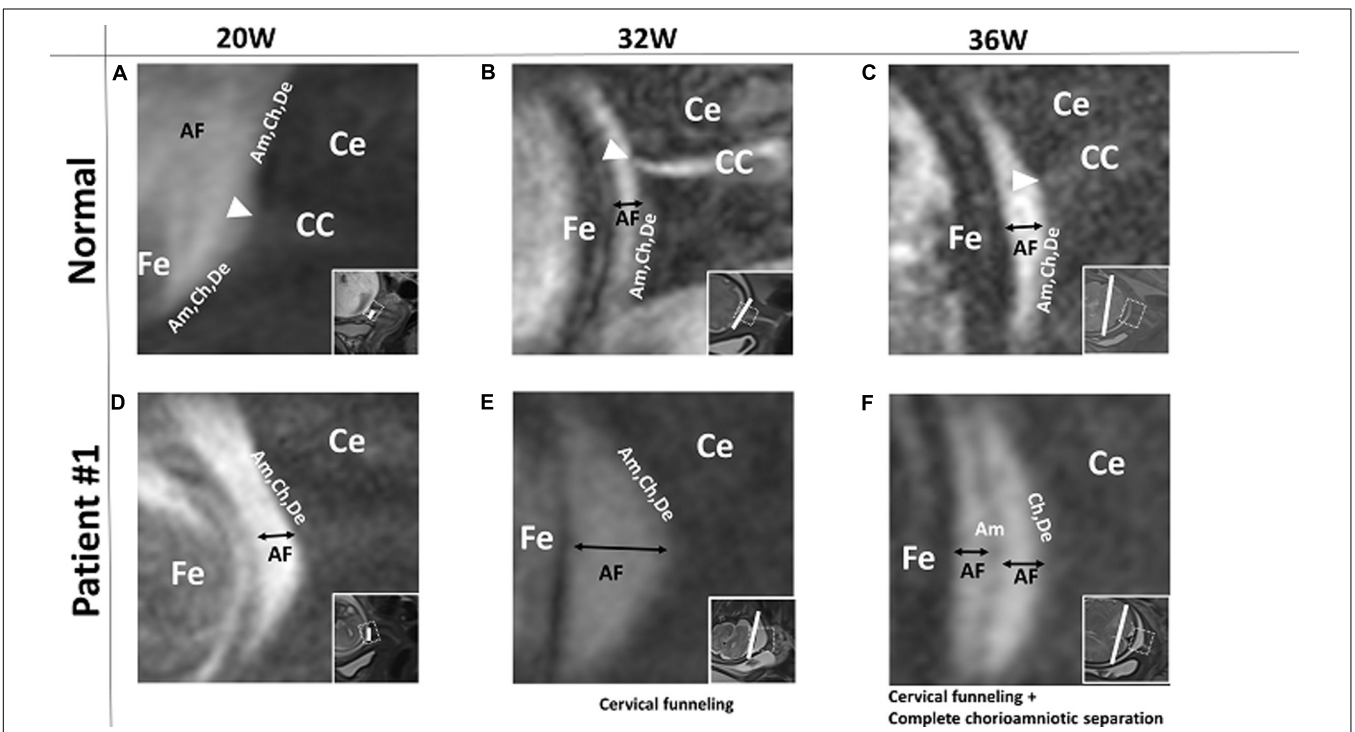

FIGURE 1 | A pregnant woman with normal FM and Patient #1. **(A–C)** 3D-CISS images from a pregnant woman with normal, intact FM at the indicated time points. The white triangle indicates FM suspended in the cervical canal region. Images from patient #1, showing normal FM at 20 weeks **(D)**, cervical funneling at 32 weeks **(E)**, and cervical funneling and complete chorioamniotic separation at 36 weeks **(F)**. Insets show T2WI images of the same regions. The white lines indicate the diameter of the cervix anatomical internal os. AF, amniotic fluid; Am, amnion; CC, cervical canal; Ce, cervix; Ch, chorion; De, decidua; Fe, fetus.

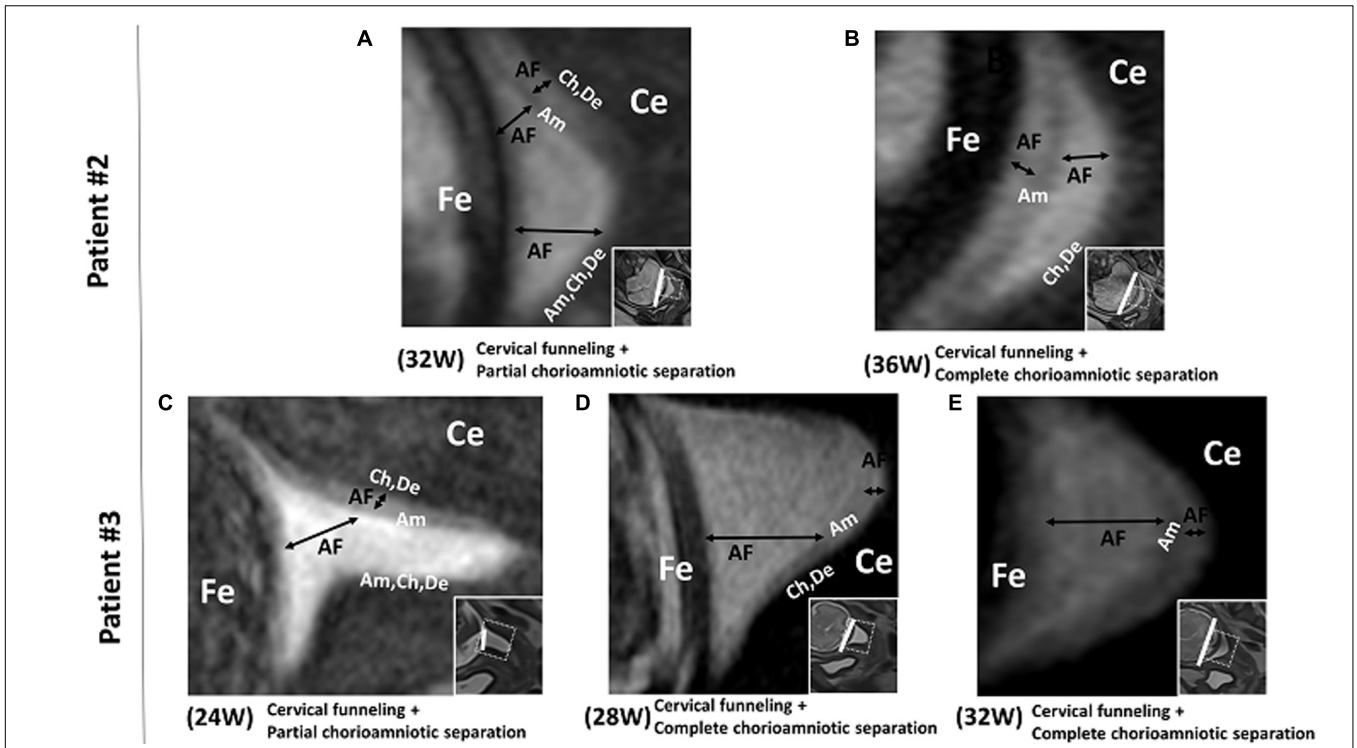

FIGURE 2 | Patients # 2 and # 3. 3D-CISS images from patient #2, showing cervical funneling and partial chorioamniotic separation at 32 weeks **(A)** and cervical funneling and complete chorioamniotic separation at 36 weeks **(B)**. Images from patient #3, showing cervical funneling and partial chorioamniotic separation at 24 weeks **(C)** and cervical funneling and complete chorioamniotic separation at 28 weeks **(D)** and 32 weeks **(E)**. Insets show T2WI images of the same regions. The white lines indicate the diameter of the cervix anatomical internal os. AF, amniotic fluid; Am, amnion; Ce, cervix; Ch, chorion; De, decidua; Fe, fetus.

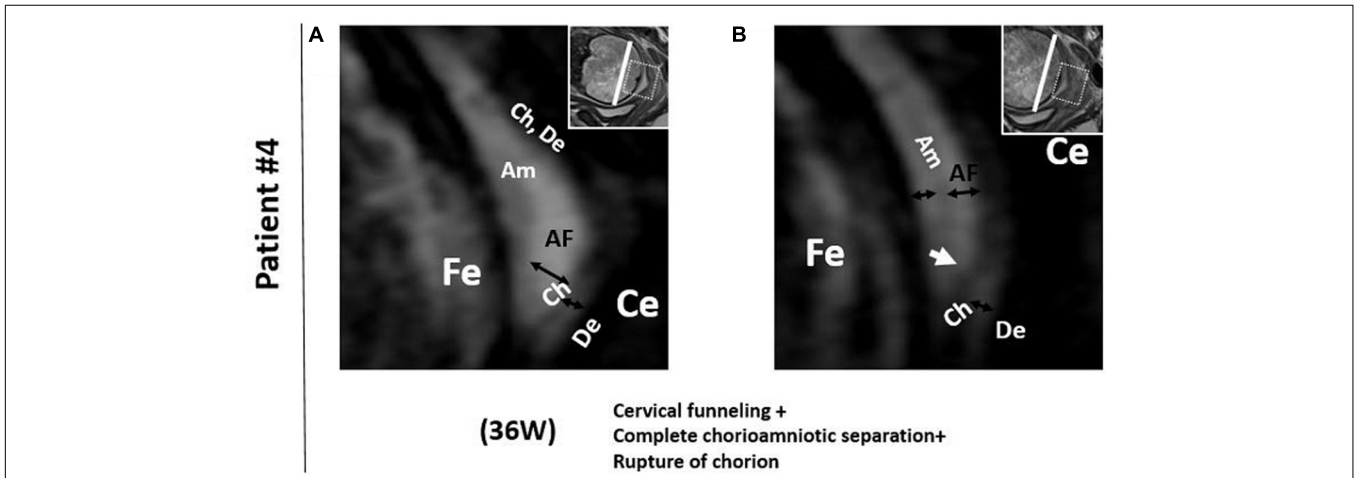

FIGURE 3 | Patient 4. 3D-CISS images from patient #4 showing cervical funneling, chorioamniotic separation, and chorionic rupture at 36 weeks. The white arrow indicates the point of chorionic rupture. Insets show T2WI images of the same regions. The white lines indicate the diameter of the cervix anatomical internal os. AF, amniotic fluid; Am, amnion; Ce, cervix; Ch, chorion; De, decidua; Fe, fetus.

Consistent with the *in vitro* studies, our *in vivo* study indicates that the stretch of FM is the first step in FM rupture. During pregnancy, outward pressure on FM from the amniotic fluid is balanced by inward pressure from the uterine wall. However, when the cervical internal os opens (cervical funneling), inward pressure on the FM overlying the cervix will decrease, and the FM will protrude into the cervical canal, causing the stretch of FM. Our longitudinal data suggest that the FM stretch in the paracervical weak zone can lead to chorioamniotic separation. Data from *in vitro* studies suggest that the mechanical force applied to FM reduces the adhesiveness between amnion and chorion, leading to chorioamniotic separation (Strohl et al., 2010). This

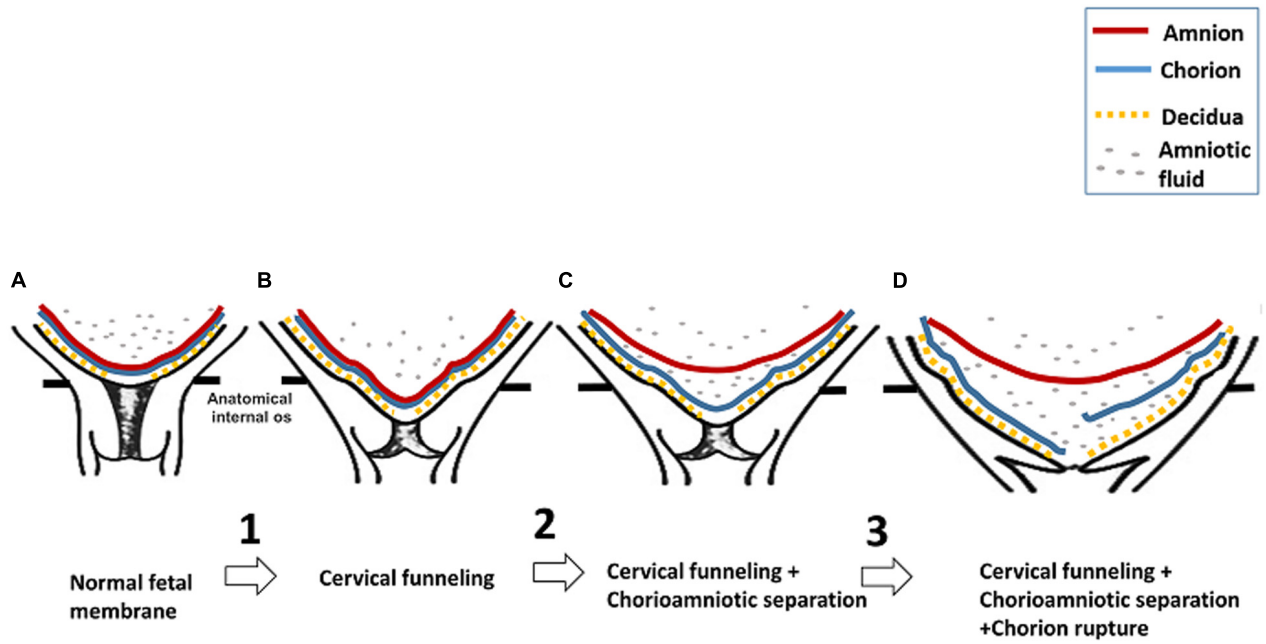

FIGURE 4 | Schematic of the first three steps of premature FM rupture detected by 3D-CISS. **(A)** After 16 weeks' gestation, the amnion (red) and chorion (blue) usually fuse, and the chorion is fused to the decidua (yellow) at the maternal–fetal interface. No amniotic fluid (gray dots) can be seen between the amnion and chorion or between the chorion and decidua. **(B)** In step 1, the FM stretches as it protrudes into the cervix when the internal cervical os dilates, causing cervical funneling. **(C)** In step 2, the amnion separates from the chorion, and amniotic fluid is detectable between the amnion and chorion. **(D)** In step 3, the FM undergoes additional stretch upon further internal cervical os dilation. This can result in chorion rupture.

result is also supported by *in vitro* second harmonic generation microscopy studies of FM, revealing that the repeated mechanical loading affects the integrity of the amnion–chorion interface and can increase the risk of FM rupture (Mauri et al., 2013).

Before 14 weeks' gestation, the chorion and amnion have not yet fused together, and the chorioamniotic separation is always normal. After 16 weeks, however, any chorioamniotic separation is identified as uncommon and anomalous (Kim et al., 2007; Bibbo et al., 2016). Such separation is dangerous, as the ultrasound-detected chorionic separation after 16 weeks is associated with adverse perinatal outcomes such as fetal extremity deformities, fetal death (Graf et al., 1997; Levine et al., 1998), and preterm delivery (Levine et al., 1998; Sydorak et al., 2002; Devlieger et al., 2003; Wilson et al., 2003). The 3D-CISS images can detect chorioamniotic separation, since the amniotic fluid lies between the chorion and amnion.

We observed that the chorioamniotic separation which occurs before FM rupture is consistent with three sets of previous data. First, in clinical observations, FM components are frequently separated at delivery after spontaneous rupture of the membranes before delivery (Strohl et al., 2010). Second, a video-recorded sequence of *in vitro* FM rupture revealed that the chorion and amnion separated before rupture (Arikat et al., 2006). Third, in *in vitro* mechanical tests, two peaks were noted in the force vs. displacement curve, suggesting that FM rupture occurs via separate rupture of the amnion and chorion (Artal et al., 1976; Lavery and Miller, 1977; Oxlund et al., 1990; Schober et al., 1994; El Khwad et al., 2005).

In patient 4, the chorion ruptured before the amnion, which is supported by *in vitro* studies (Artal et al., 1976; Lavery and Miller, 1979; Helmig et al., 1993; Oyen et al., 2004; Arikat et al., 2006).But some studies suggest that the amnion ruptures first (Artal et al., 1976; Lavery and Miller, 1979; Helmig et al., 1993; Oyen et al., 2004; Arikat et al., 2006). Our data are consistent with *in vitro* mechanical testing revealing that the amnion was consistently stronger, stiffer, and more ductile than the chorion (Arikat et al., 2006). The amnion may be stronger because it is composed of a dense layer of collagen fibrils, where the FM strength mainly comes from (Strauss, 2013).

The major strength of this work is the first ever use of 3D-CISS MRI to obtain *in vivo* images of the FM at much higher contrast and better resolution than other types of MRI or ultrasound. Clinical ultrasound is a series of 2D images acquired at several limited angles, which cannot provide a 3D description of the FM overlying the cervix. In comparison, 3D-CISS MRI is not operator dependent and can provide a high resolution, high contrast 3D spatial coverage of FM with multi-planar viewing angle capability. Therefore, 3D-CISS MRI provides a novel way to study the FM overlying the cervix. Additionally, by longitudinally imaging patients, we could define the sequences of events leading to FM rupture.

In this study, we used a 3.0 T MRI to image the FM of pregnant women. MRI has been used to evaluate obstetrical, placental, and fetal abnormalities in pregnant patients for more than 30 years, and its application during pregnancy is generally considered safe for the fetus (Patenaude et al., 2014; Radiology TACo, 2015;

Ray et al., 2016). Compared with the current commonly used fetal MRI sequence, the 3D-CISS sequence was applied without exceeding either of the specific absorption rate and acoustic noise. Additionally, 3D-CISS is a high-speed sequence (4 min) and therefore reduced the patients' exposure to the magnetic.

Our study has three main limitations. First, we had a small sample size and our data are qualitative in nature. Second, we did not measure other FM characteristics such as thickness and signal intensity. Lastly, our medical records did not separate PPROM from PTL in the history of preterm delivery.

CONCLUSION

In summary, our data support the *in vitro* model that the FM ruptures according to a sequence starting with stretch of the chorion and amnion together, then separation of the amnion from the chorion, next the rupture of the chorion, and finally the rupture of the amnion ruptures. An important next step is to conduct a larger longitudinal study to confirm these findings. If we can define an MRI marker that predicts FM rupture, we may be able to intervene to prevent PPROM.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary files.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Washington University in St. Louis Institutional Review Board (protocols 201612140 and 201707152). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WQ and PZ designed the experiment. WQ and WiW evaluated magnetic resonance images. ZS, XM, HW, WnW, ZW, ZK-W, PW, and QW collected the data and aided in preparation of the manuscript. RM co-supervised the research. YW obtained funding for the project, supervised the work, and participated in preparation of the manuscript.

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Occurrence of a RAGE-Mediated Inflammatory Response in Human Fetal Membranes

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Context: Sterile inflammation has been shown to play a key role in the rupture of the fetal membranes (FMs). Moreover, an early and exacerbated runaway inflammation can evolve into a preterm premature rupture of membranes and lead to potential preterm birth. In this context, we investigated the receptor for advanced glycation end products (RAGE), an axis implied in physiological sterile inflammation, in conjunction with two major ligands: AGEs and High-Mobility Group Box 1 (HMGB1). Our first objective was to determine the spatiotemporal expression profiles of the different actors of the RAGE-signaling axis in human FMs, including its intracellular adaptors Diaphanous-1 and Myd88. Our second goal was to evaluate the functionality of RAGE signaling in terms of FMs inflammation.

Methods: The presence of the actors (RAGE, HMGB1, Myd88, and Diaphanous-1) at the mRNA level was investigated by reverse transcription quantitative polymerase chain reaction (RT-qPCR) in the human amnion and choriodecidua at the three trimesters and at term. Measurements were conducted at two distinct zones: the zone of intact morphology (ZIM) and the zone of altered morphology (ZAM). Then, proteins were quantified using Western blot analysis, and their localization was evaluated by immunofluorescence in term tissues. In addition, pro-inflammatory cytokine secretion was quantified using a Multiplex assay after the treatment of amnion and choriodecidua explants with two RAGE ligands (AGEs and HMGB1) in the absence or presence of a RAGE inhibitor (SAGEs).

Results: The FMs expressed the RAGE-signaling actors throughout pregnancy. At term, RNA and protein overexpression of the RAGE, HMGB1, and Diaphanous-1 were found in the amnion when compared to the choriodecidua, and the RAGE was overexpressed in the ZAM when compared to the ZIM. The two RAGE ligands (AGEs and HMGB1) induced differential cytokine production (IL1β and TNFα) in the amnion and choriodecidua.

Conclusion: Considered together, these results indicate that RAGE signaling is present and functional in human FMs. Our work opens the way to a better understanding of FMs weakening dependent on a RAGE-based sterile inflammation.

Keywords: fetal membranes, RAGE, alarmins, sterile inflammation, rupture of fetal membranes

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INTRODUCTION

Fetal membranes are an essential actor in human parturition; if they do not achieve their missions, the childbirth can be impacted (Naeye and Peters, 1980; Romero et al., 2006; Menon, 2016; Menon and Richardson, 2017). These fetal tissues consist of two layers: the amnion, which is the innermost layer directly in contact with the amniotic fluid (AF), and the chorion, which adheres to the maternal decidua. This 9-month organ participates in the correct development of the fetus by providing AF homeostasis as well as physical and microbial barriers during pregnancy; however, they also play a role in parturition by their programmed rupture at term (after 37 weeks gestation) (Buhimschi et al., 2004; Moore et al., 2006; King et al., 2007; Prat et al., 2012). In this way, FMs undergo progressive weakening leading to this physiologic rupture of membranes (ROM) thanks to several mechanisms, such as apoptosis, senescence, or inflammation (Parry and Strauss, 1998; Menon et al., 2019).

Recently, an increasing number of studies have shown the implication of one key phenomenon in the FMs weakening: sterile inflammation (Girard et al., 2014; Romero et al., 2014, 2015). This concept is dependent on specific molecules called alarmins or "damage-associated molecular patterns" (DAMPs), which are released and recognized by pattern recognition receptors (PRRs) leading to a microbial-free inflammatory response or a "sterile" inflammation. Examples of DAMPs include high-mobility group box 1 (HMGB1) protein, the S100 protein family, uric acid, cell-free DNA, and advanced glycation end-products (AGEs), and examples of PRRs include toll-like receptors, scavenger receptors, NOD-like receptors, and the receptor for AGEs (Taglauer et al., 2014; Nadeau-Vallée et al., 2016; Brien et al., 2019). It has been determined that AF contains many of these alarmins, which induce pro-inflammatory cytokine release by activating various cellular pathways (Holmlund et al., 2007; Jakobsen et al., 2012; Bredeson et al., 2014; Menon and Moore, 2020). Lappas and colleagues demonstrated an induction of cytokine release (IL1β, IL6, IL8, TNFα) by FMs in response to AGEs (Lappas et al., 2007).

However, it still remains unclear how this phenomenon works exactly or which receptor translates this inflammatory signaling to the FMs to prepare for a successful ROM that does not occur before 37 weeks. In the case of early activation, preterm prelabor rupture of the membranes (pPROM) can occur. pPROM affects 3–4% of all pregnancies and leads to 30–40% of all preterm births. Yearly, there are about 15 million cases of preterm birth worldwide. It is important to note that this problem is associated with the rise of perinatal mortality, morbidity, and developmental troubles (Schreiber and Benedetti, 1980; Silverman and Wojtowycz, 1998; Fujimoto et al., 2002; England et al., 2013; Lorthe, 2018; Bouvier et al., 2019; Shiqiao et al., 2019). Thus, it is essential to better understand the ROM to improve pPROM diagnostics and clinical care.

In this study, we decided to investigate the implication of one actor: RAGE (Neeper et al., 1992; Brett et al., 1993). Originally discovered in 1992 as a new member of the immunoglobulin superfamily of receptors, the RAGE is a 55 kDa cell surface receptor that interacts with several ligands (including AGEs and HMGB1) implicated in the pathogenesis of many inflammatory diseases (Kierdorf and Fritz, 2013; Ray et al., 2016; Hudson and Lippman, 2018). Indeed, the RAGE is known to activate pro-inflammatory pathways and the release of cytokines and has been described as participating in the weakening of FMs (Rzepka et al., 2015). Plus, lower concentrations of a soluble RAGE, a competitive RAGE isoform lacking the intracellular domain, has been discovered in the maternal serum of patients suffering from pPROM (Hájek et al., 2008). Furthermore, even if the expression of the RAGE has been outlined in the placental sphere, little is known about the RAGE in FMs and even less on the action and physiopathology of the RAGE in terms of the ROM (Yan et al., 2018). Plus, it is well known that RAGE signaling activity relies on its interaction with intracellular adaptors proteins such as Diaphanous-1, Myd88, and TIR adaptor protein (TIRAP) (Hudson et al., 2008; Sakaguchi et al., 2011). Thus, this study intends to provide more information about the RAGE axis actors in the FMs and determine if a RAGE-dependent inflammatory response can specifically occur in the amnion or choriodecidua when exposed to alarmins such as HMGB1 and AGEs.

MATERIALS AND METHODS

Chemicals

HMGB1 (SRP6265, 10 μg/mL in phosphate-buffered saline 1X) was purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France) and AGE-bovine serum albumin (10 mg/mL, ab51995) from Abcam (Paris, France). Semi-synthetic glycosaminoglycan ethers (SAGEs) (GM-1111, 10 mg/mL in water), used for the RAGE inhibition, were kindly gifted by GlycoMira Therapeutics (Salt Lake City, UT, United States) (Zhang et al., 2011). Cell culture medium and antibiotics (streptomycin, penicillin, amphotericin B) were obtained from Fisher Scientific (Illkirch-Graffenstaden, France). Fetal bovine serum (FBS) was purchased from Eurobio Scientific (Les Ulis, France). Collagen I was obtained from Stemcell Technologies (Grenoble, France). Superscript IV first-strand-synthesis system, Taq DNA polymerase recombinant (10342020), and Pierce BCA protein assay kit (23225) were obtained from Fisher Scientific.

Tissue Collection

Full-term FMs were collected from non-smoking women with healthy pregnancies from vaginal or scheduled cesarean deliveries (breech presentation, scarred wombs) (Centre Hospitalier Universitaire Estaing, Clermont-Ferrand, France) after obtaining informed consent. Gestational ages were 39.08 ± 0.11 weeks, mean maternal ages were 35.30 ± 0.94 years, and maternal body mass index (BMI) was 26.64 ± 6.65. The selected FMs were collected from singleton pregnant women who had no underlying diseases and no gestational diabetes or clinical chorioamnionitis (defined by maternal fever, uterine tenderness, and/or purulent amniotic fluid). The research protocol was approved by the institutional regional ethics committee (DC-2008-558). The

amnion was dissociated from the choriodecidua. The zone of altered morphology (ZAM, with the thread) and the zone of intact morphology (ZIM, away from the thread) were also distinguished. Indeed a suture sewn placed onto the FMs (from cesarean deliveries) in front of the cervix by the midwife allowed us to identify ZAM; then, a 4-cm-diameter circle was cut and considered as ZAM, and explants localized places away from circle boundary were considered as ZIM.

Concerning samples used for RAGE axis actor exploration throughout the pregnancy, first-trimester membranes (N = 3) were obtained following aspiration after voluntary termination of pregnancy. Second-trimester membranes were harvested after medical termination of pregnancy (N = 3). Eligible cases corresponded to lethal fetal anomalies that had no impact on the FMs (e.g., severe cardiac anomalies or brain damage). Then, preterm third-trimester membranes (N = 3) were collected from pregnancies after cesarean births. The amnion was dissociated from the choriodecidua except for trimester 1 samples.

Tissue Culture

Explants (dissociated) of the amnion and choriodecidua were cultivated (5% CO_2, 95% humidified air, 37°C) in Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM-F12- GlutaMAX) supplemented with 10% FBS, 100 μg/ml of streptomycin, 100 U/ml of ampicillin, and 25 μg/ml amphotericin B. Explants were 2 cm^2 in size, obtained 2 cm away from the pre-placental edge and prepared by dissection. Tissue fragments were transferred (in duplicate) to 24-well culture plates and incubated in cell media at 37°C for 1 h before treatment.

Tissue Explant Treatment

Explants were treated with AGEs (150, 250, and 500 μg/ml) or HMGB1 (100, 200, and 300 ng/ml) in the absence or presence of SAGEs (500 μg/ml) for 18 h (cell medium collection for cytokine release assay). In addition, an internal control was performed by treating explants with a combination of lipopolysaccharide (LPS) (10 μg/ml) and TNFα (100 ng/ml) to validate inflammatory reactivity of FMs samples used. FMs were validated when there was a release response of at least one cytokine.

RT-PCR and Quantitative RT-PCR

After the disruption step with Precellys homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) using ceramic beads (KT03961, Ozyme, Saint-Cyr-l'École, France), total RNAs were extracted from human amnion or choriodecidua using RNAzol® RT (RN190, Molecular Research Center, Cincinnati, OH, United States). The reverse transcription was made from 1 μg of RNA using a Superscript IV first-strand-synthesis system for reverse transcription polymerase chain reaction (RT-PCR). PCR experiments were performed using specific oligonucleotides (**Table 1**). Results were analyzed on a 2% agarose gel and verified by DNA sequencing. RAGE, HMGB1, Myd88, and Diaphanous-1 expression was assessed by quantitative RT-PCR (RT-qPCR) performed using LightCycler® 480 SYBR Green I Master (Roche, Meylan, France). Transcript quantification was performed twice on at least four independent experiments. Results were normalized to the geometric mean of the human housekeeping genes RPL0 (36b4) and RPS17 (acidic ribosomal phosphoprotein P0 and ribosomal protein S17, respectively) as recommended by the MIQE guidelines (Bustin et al., 2009).

Western Blot Analysis

After the preliminary tissue homogenization previously described, total proteins were extracted from human amnion and choriodecidua (total, ZIM, or ZAM) with a plasma membrane protein extraction kit (BioVision, Lyon, France), and protein sample concentrations were measured using a Pierce BCA protein assay kit. For Western blot analysis, proteins were resolved on a 4–15% Mini-PROTEAN® TGX Stain-Free™ Precast Gel (Bio-Rad, Marnes-la-Coquette, France) to perform total protein normalization (Gilda and Gomes, 2013). Before transfer, stain-free imaging was completed. This technology utilizes a proprietary trihalo compound to enhance natural protein fluorescence by covalently binding to tryptophan residues with a brief UV activation (Bio-Rad). Then, the transfer was performed on nitrocellulose membrane (Bio-Rad) and saturated over 1 h 30 min with 5% skimmed milk in tris-buffered saline (TBS) 1X. Antibody against the RAGE (1/1000, AF1179, R&D Systems, Noyal-Châtillon-sur-Seiche, France), HMGB1 (1/10000, ab79823, Abcam), Myd88 (1/1000, ab133739, Abcam), and Diaphanous-1 (1/5000, ab1173, Abcam) were diluted in 5% skimmed milk-TBS 1X-TWEEN® 20 0.1% and incubated overnight at 4°C. The next day, the membrane was washed three times with TBS 1X/TWEEN® 20 0.1% and incubated at room temperature with a horseradish peroxidase coupled secondary antibody anti-goat or anti-rabbit (1/5000 or 1/10,000, respectively, BI 2403 or BI 2407, Abliance, Compiègne, France) for 1 h 30 min. The revelation was completed using an ECL clarity kit for Western blot on the ChemiDoc™ imaging system (Bio-Rad). Image Lab Software (Bio-Rad) was used for quantification. Results are expressed as a mean of at least three independent experiments.

Supernatant Protein Concentration

Before the Multiplex assays were completed, the supernatants of the treated explants were concentrated into 2 kDa centrifugal filter units (Vivacon® 500, Sartorius, Aubagne, France) for protein concentration and purification, following the manufacturer's instructions.

Cytokine Multiplex Assay

The release of TNFα, IL1β, IL6, and IL8 in the culture media was tested using a MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel Milliplex® MAP Kit (Merck Millipore, Molsheim, France) based on the Luminex® xMAP® technology, according to the manufacturer's instructions (Biosource International). Finally, cytokine concentrations were normalized to total protein concentration, and the ratio "treated/untreated" was reported.

Cellular Distress Determination

For the evaluation of the treatment impact on cell suffering, the release of the intracellular enzyme lactate dehydrogenase

TABLE 1 | Forward and reverse primer sequences used for RT-PCR and RT-qPCR amplification of human genes.

Gene	Sequence 5′-3′ (F: forward, R: reverse)	Product size (bp)	Hybridation temperature (°C)
hsRAGE	F:TGTGCTGATCCTCCCTGAGA R: CGAGGAGGGGCCAACTGCA	139	61
hsRPL0/36B4	F: AGGCTTTAGGTATCACCACT R: GCAGAGTTTCCTCTGTGATA	219	61
hsRSP17	F: TGCGAGGAGATCGCCATTATC R: AAGGCTGAGACCTCAGGAAC	169	61
hsMyd88	F: GCAGGAGGAGGCTGAGAAGC R: CGGATCATCTCCTGCACAAACT	167	63
hsDia-1	F: AGAGCCACACTTCCTTTCCATC R: TCAATCTCAATCTGGAGGTGCC	167	61
hsHMGBI	F: ACCTATATCCCTCCCAAAGGG R: TTTTTGGGCGATACTCAGAGCA	109	61

(LDH) into the cell media was quantified on a machine automate (Siemens Vista, Paris, France) using an enzymatic assay, following the manufacturer's recommendations.

Immunofluorescence

After permeabilization in PBS 1X/FBS 10%/Triton 0.1% over 1 h 30 min, the primary antibody against the RAGE (1/100, ab37647, Abcam) was applied on the FM sections overnight at 4°C. After three washes in permeabilization buffer, secondary antibody anti-rabbit Alexa Fluor 488 (1/1000, A21206, Life Technologies) was incubated for 2 h at room temperature. Slides were washed three times in TWEEN® PBS 1X and incubated with Hoechst (15 min, dilution in PBS 1X 1/10,000; bisBenzimide H, 33258, Sigma-Aldrich). Finally, slides were mounted with CitiFluor^{TM} Tris-MWL 4–88 (Electron Microscopy Science) and examined under an Apotome Zeiss Imager microscope (magnification $\times$200). For negative controls, incubation without the primary antibody was performed.

Statistical Analysis

The data expressed as mean $\pm$ standard error of the mean (*SEM*) are an average of duplicates or triplicates of at least three independent experiments. The comparison of means was performed by non-parametric test (Kruskal–Wallis one-way ANOVA test followed by a Dunn's post-test for comparison of more than two conditions), or a Wilcoxon signed-rank test (comparing a fold change to one) using PRISM software 5.02 (GraphPad Software Inc.). For all studies, values were considered significantly different at $p < 0.05$(*), $p < 0.01$(**), and $p < 0.001$(***).

RESULTS

Are RAGE Axis Actors Expressed in Fetal Membranes During Pregnancy?

We investigated the mRNA expression profile of the RAGE, its adaptors and one ligand (HMGB1) in FMs on amnion and choriodecidua samples throughout pregnancy (first trimester: 1 to 13 weeks of gestation (WG); second trimester: 14–26 WG; third trimester: 27–37 WG; at term: 38–40 WG, by cesarean or vaginal delivery). RT-PCR experiments revealed that FMs expressed the RAGE, HMGB1, Myd88, and Diaphanous-1 in both layers, the amnion and choriodecidua in each of the stages considered (**Figure 1A**). No significant difference in RAGE expression was revealed by RT-qPCR between trimesters (data not shown). Plus, RAGE protein expression was also demonstrated in both layers obtained after vaginal delivery or caesarean section with the separate consideration of the ZIM and ZAM (**Figure 1B**).

Is the RAGE Axis Actor Expression Layer- or Zone-Dependent at Term?

Based on RT-qPCR analysis at term, we revealed an overexpression of RAGE (**Figure 2A**, left panel) and HMGB1 (**Figure 2A**, right panel) in the amnion compared to the choriodecidua in consideration of the ZAM. Moreover, RAGE expression was also area-dependent: it was found to be significantly more expressed in the rupture zone (ZAM) than in the ZIM. These results were confirmed at the protein level for both using western blot analysis (**Figure 2B**, upper panel for representative experiment and **Figure 2B**, lower panel for quantification).

Furthermore, considering that the RAGE requires intracellular adaptor binding to induce a cellular response, we also investigated the expression of Diaphanous-1, Myd88, and TIR adaptator protein (TIRAP). First, TIRAP was found at very low levels for mRNA and not detected by immunoblot in both layers (data not shown). Furthermore, we revealed that Diaphanous-1 is overexpressed in the amnion for mRNA (only in ZAM) and protein level in both zones (**Figures 3A,B**, left panels). Plus, we found a Myd88 protein overexpression in the choriodecidua in comparison to the amnion in the ZAM (**Figures 3A,B**, right panels).

Are the Amnion or Choriodecidua Able to Trigger an Inflammatory Response to Alarmins?

Before further investigation, cell toxicity that may have been caused by the induction of AGEs and HMGB1 treatments in the amnion and choriodecidua explants was checked. This was done using LDH release measurements in the culture media after 18 h of alarmin treatment and revealed no cell toxicity for each condition (three concentrations tested for each one; **Figure 4**). Then, we performed a dose response effect of AGEs and HMGB1 on cytokine release after 18 h

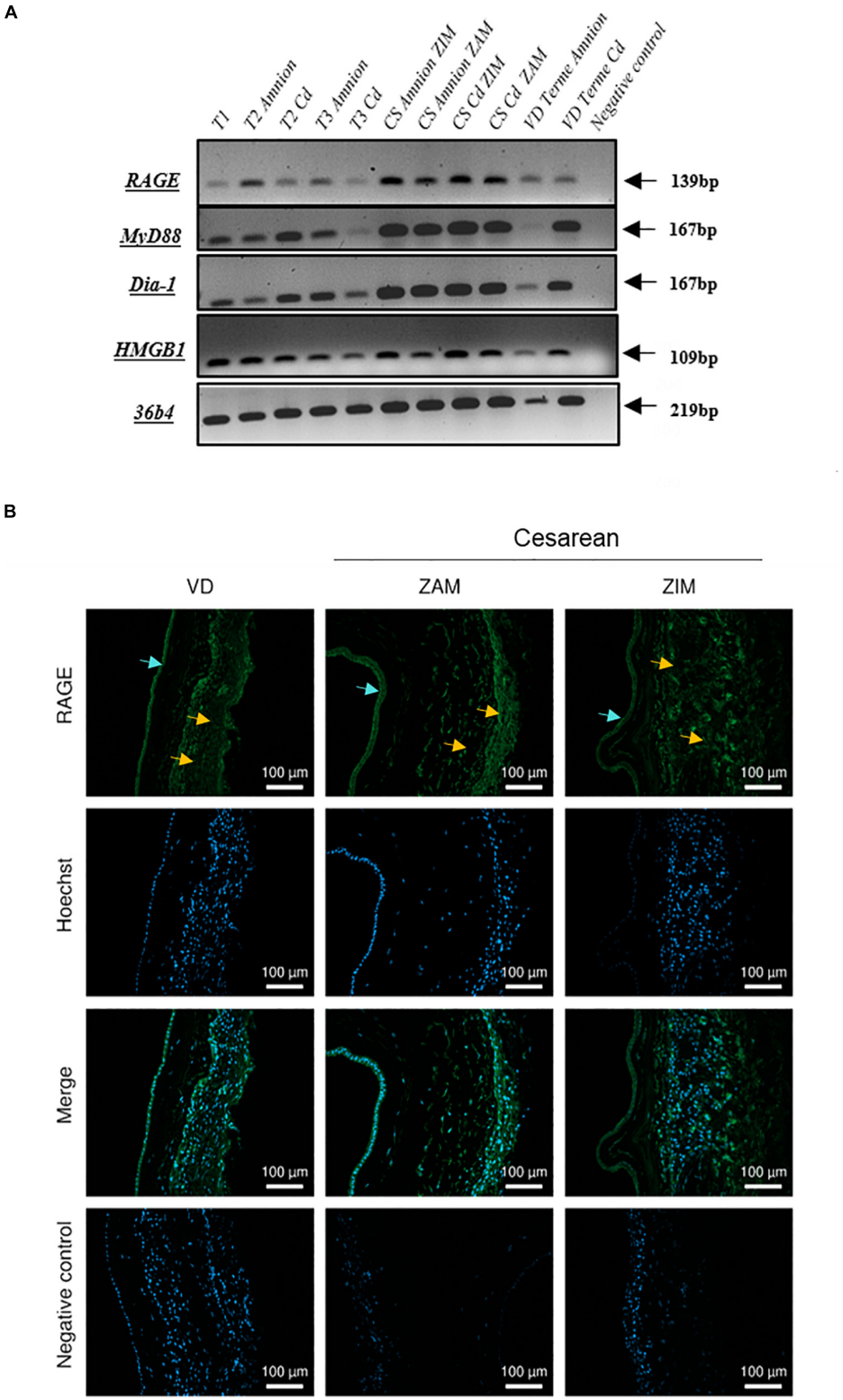

FIGURE 1 | Expression of RAGE-signaling actors in human fetal membranes. **(A)** RNA expression of the RAGE, Myd88, Diaphanous-1, and HMGB1 was detected by RT-PCR on the amnion and choriodecidua (Cd) samples from the different trimesters (T1, T2, and T3), caesarean (CS), or vaginal (VD) delivery at term. Negative controls were performed in the absence of cDNA. **(B)** RAGE protein localization in human fetal membranes at term was investigated by immunofluorescence (green staining, Alexa488) on sections from vaginal delivery (VD) or caesarean (ZIM and ZAM) at magnification ×200. Cyan arrows indicate amniotic epithelium and yellow designate choriodecidua. Nuclei were counterstained with Hoechst (blue). Negative controls consisted of primary antibody-free incubation.

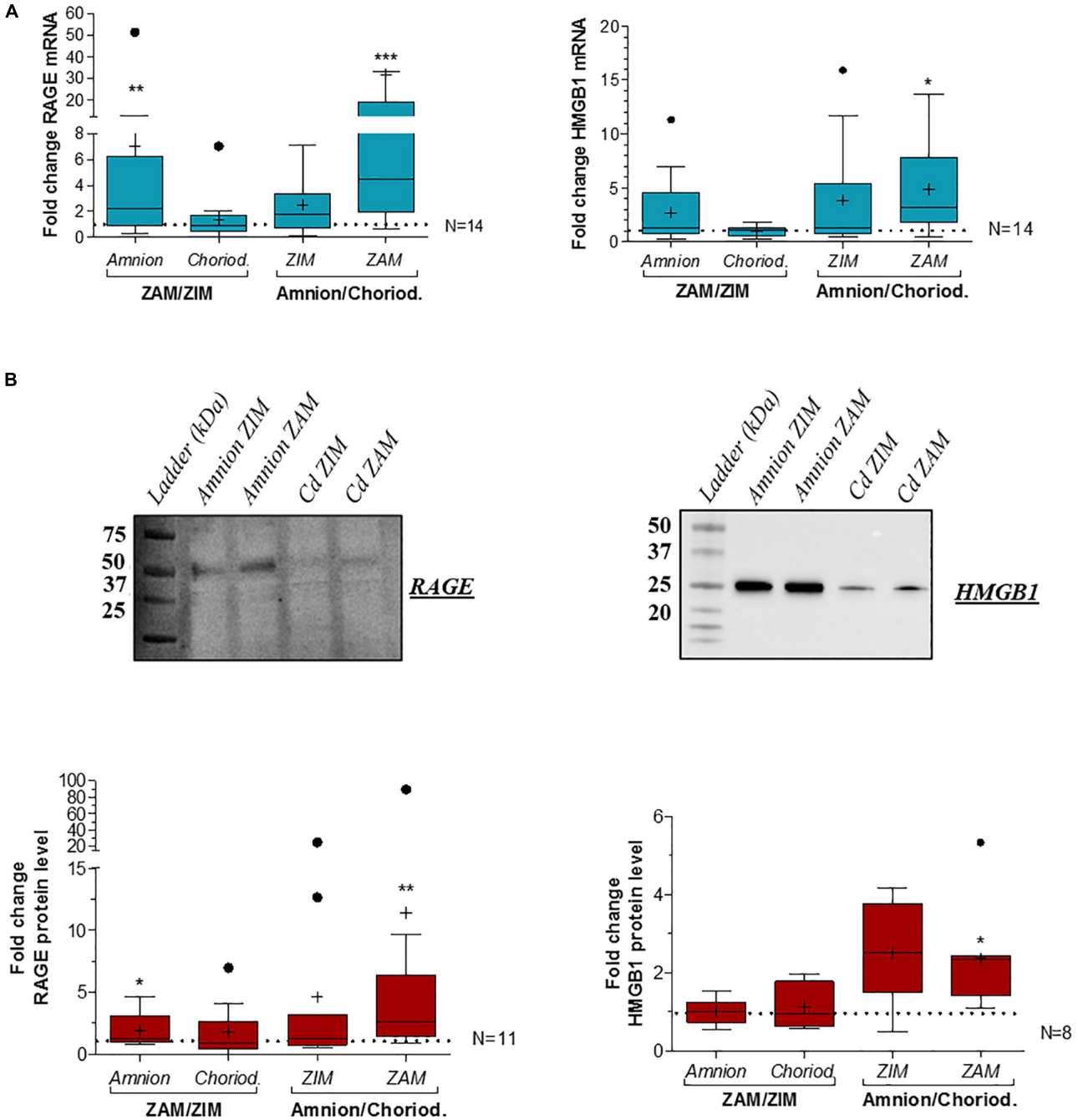

FIGURE 2 | Quantification of RAGE and HMGB1 expression in human fetal membranes at term in the ZIM and ZAM. RAGE and HMGB1 expression were quantified by RT-qPCR ($N = 14$) **(A)** and Western blot (respectively, $N = 11$ and $N = 8$) **(B)** on the amnion and choriodecidua (Cd) at term in the ZIM and ZAM. To highlight an area (ZAM vs. ZIM) or tissue (Amnion vs. Choriodecidua) effect, ZAM is reported on ZIM (ZAM/ZIM in left panel) in both amnion and choriodecidua, and amnion is reported on choriodecidua (amnion/choriodecidua in right panel) in both ZIM and ZAM. Statistical fold change analysis was performed by a Wilcoxon signed-rank test comparing to one. * means $p < 0.05$, ** means $p < 0.01$, *** means $p < 0.001$. Results are presented in Tukey boxes, and means are indicated by "+." Representative Western blot membranes indicate the band that was quantified proteins (RAGE: 50 kDa and HMGB1 at 25 kDa).

of treatment on both the amnion and choriodecidua. First, in the amnion (**Figure 5**, upper left panel), we observed that AGEs did not stimulate IL8 release, but increased TNFα in the same way for all concentrations (150, 250, and 500 μg/ml) and IL1β (more at 150 than 500 μg/ml). Finally, we found that AGEs also induced IL6 release at 150 μg/ml. For the choriodecidua (**Figure 5**, upper right panel), the same responses as the amnion were found for IL8 and TNFα, and IL1β was

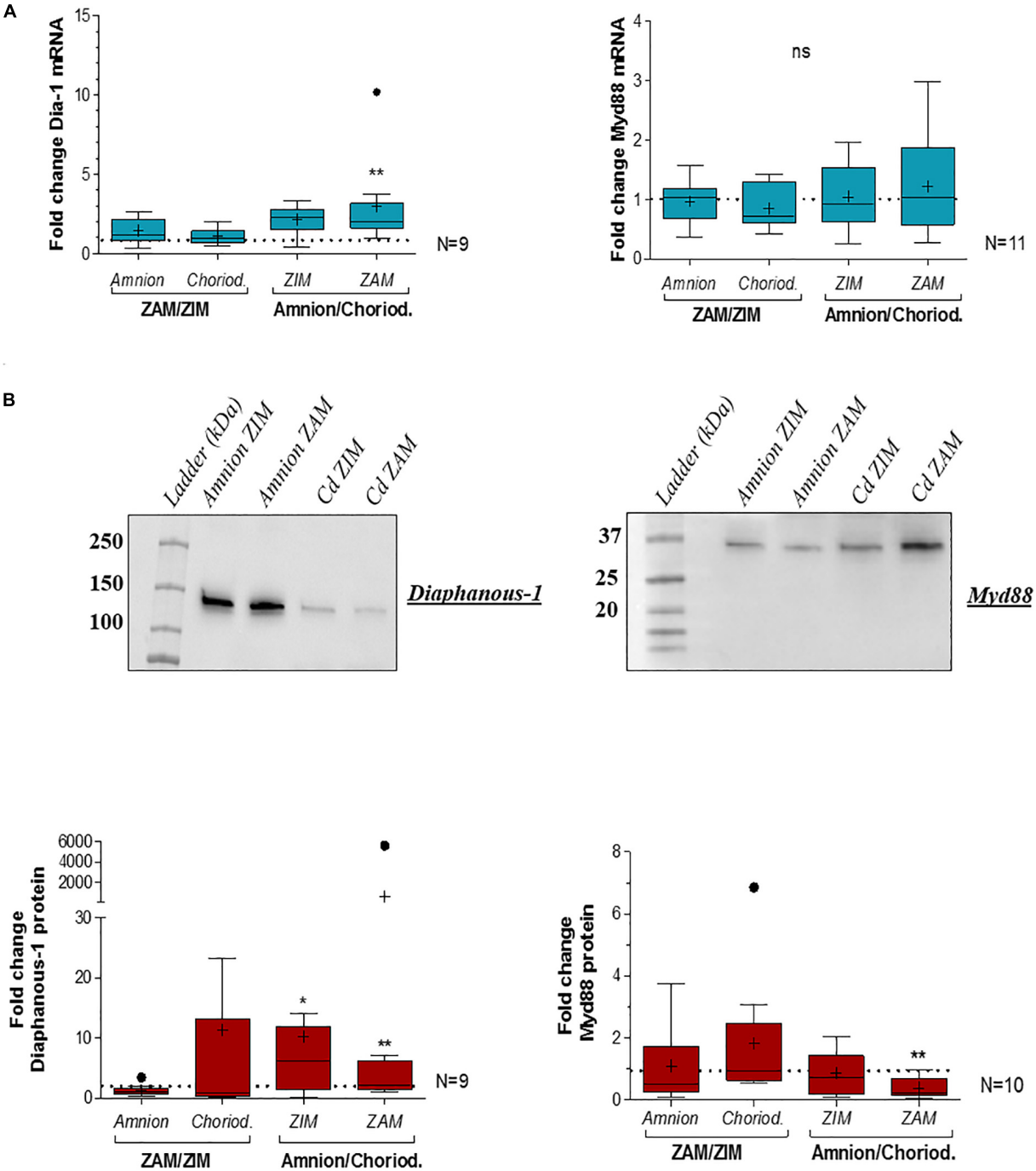

FIGURE 3 | Quantification of RAGE-signaling adaptors in human fetal membranes at term in the ZIM and ZAM. Myd88 and Diaphanous-1 expressions were quantified by RT-qPCR (respectively, $N = 11$ and $N = 9$) **(A)** and Western blot ($N = 9$ and $N = 10$) **(B)** on the amnion and choriodecidua (Cd) at term in the ZIM and ZAM. To highlight an area (ZAM vs. ZIM) or tissue (amnion vs. choriodecidua) effect, ZAM is reported on ZIM (ZAM/ZIM in left panel) in both amnion and choriodecidua, and amnion is reported on choriodecidua (amnion/choriodecidua in right panel) in both ZIM and ZAM. Statistical fold change analysis was performed by a Wilcoxon signed-rank test comparing to one. * means $p < 0.05$, ** means $p < 0.01$. Results are presented in Tukey boxes, and means are indicated by "+." Representative Western blot membranes indicate the band that was quantified (Diaphanous-1 was detected around 150 kDa and Myd88 at 37 kDa).

increased regardless of the dose (more with 500 μg/ml). By contrast, induction was not relevant for IL6 at any concentration. A second time, we demonstrated that HMGB1 (**Figure 5**, lower panel) stimulated TNFα release in both tissues (at 200 and 300 ng/ml) and IL1β at the same doses but only in the amnion. Any significant induction could be reported by

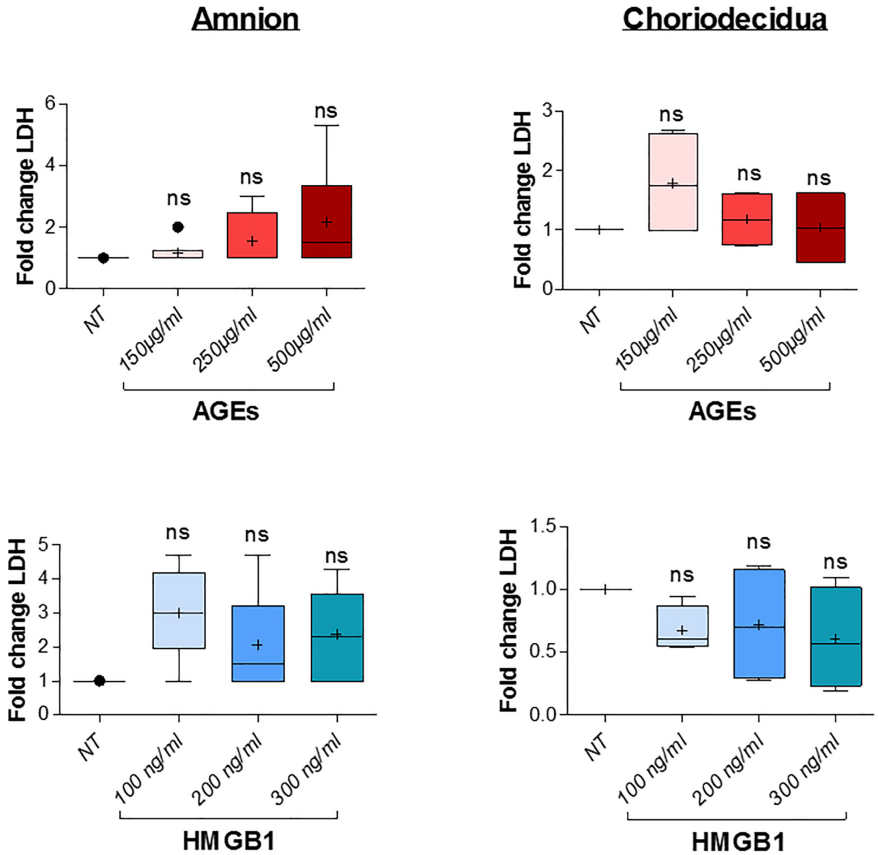

FIGURE 4 | AGES or HMGB1 treatment effects on cell toxicity in the amnion and choriodecidua explants. Toxicity was evaluated by LDH release measurement in culture supernatants after 18 h of treatment with a dose effect of AGEs (150, 250, and 500 µg/ml) or HMGB1 (100, 200, and 300 ng/ml; *N* = 3 in duplicate). Statistical analysis was performed using a Kruskal–Wallis one-way ANOVA test followed by a Dunn's post-test and showed no significant difference. Results are presented in Tukey boxes, and means are indicated by "+."

HMGB1 treatment for IL8 and IL6. Regarding our results, and in accordance with those already described in previous articles (Lappas et al., 2007; Plazyo et al., 2016), 500 µg/ml of AGEs and 200 ng/ml of HMGB1 were kept for the following SAGEs blocking experiments.

Does Blocking the RAGE Modulate the Inflammatory Response Induced by Alarmins in Fetal Membranes?

Finally, to investigate whether AGEs and HMGB1 alarmins induce a RAGE-dependent inflammatory response, we measured pro-inflammatory cytokine release in the amnion and choriodecidua co-treated with or without SAGEs (a RAGE inhibitor) for 18 h. Results demonstrated a significant lower TNFα release induction by AGEs (**Figure 6**, upper panel) and HMGB1 (**Figure 6**, lower panel) when the RAGE was inhibited by SAGEs in the amnion and choriodecidua. Plus, we noticed that AGEs and HMGB1 stimulated IL1β secretion in both layers, but in the presence of SAGEs, this induction was not any more significant for HMGB1. Finally, we found no impact on either IL8 or IL6.

DISCUSSION

Since these last years, more and more studies have underlined the importance of sterile inflammation in the weakening of FMs as a key event of the ROM (hopefully after 37 weeks of gestation). It is now considered that AF is a source of specific molecules called alarmins (or DAMPs), which are endogenously expressed and produced when cells are suffering. For example, HMGB1 is normally a nuclear protein implicated in DNA reparation, but when cells are in danger, HMGB1 is released and becomes an alarmin, triggering an inflammatory cascade. As another kind of DAMPs, AGEs are formed by the non-enzymatic Maillard reaction, between sugars and proteins, lipids, or nucleic acids (John and Lamb, 1993), and many inflammatory diseases are linked to an accumulation of these AGEs in tissues (Kang et al., 2012; Guedes-Martins et al., 2013; Wautier et al., 2014). Above all, both AGEs and HMGB1 have been described as activating inflammatory response in gestational tissues (placenta, FMs, umbilical cord) and were found to be increased in cases of pPROM. Indeed, HMGB1 has been found to be more elevated in the AF due to a release caused by damaged FMs occurring during intra-amniotic inflammation found during preterm birth

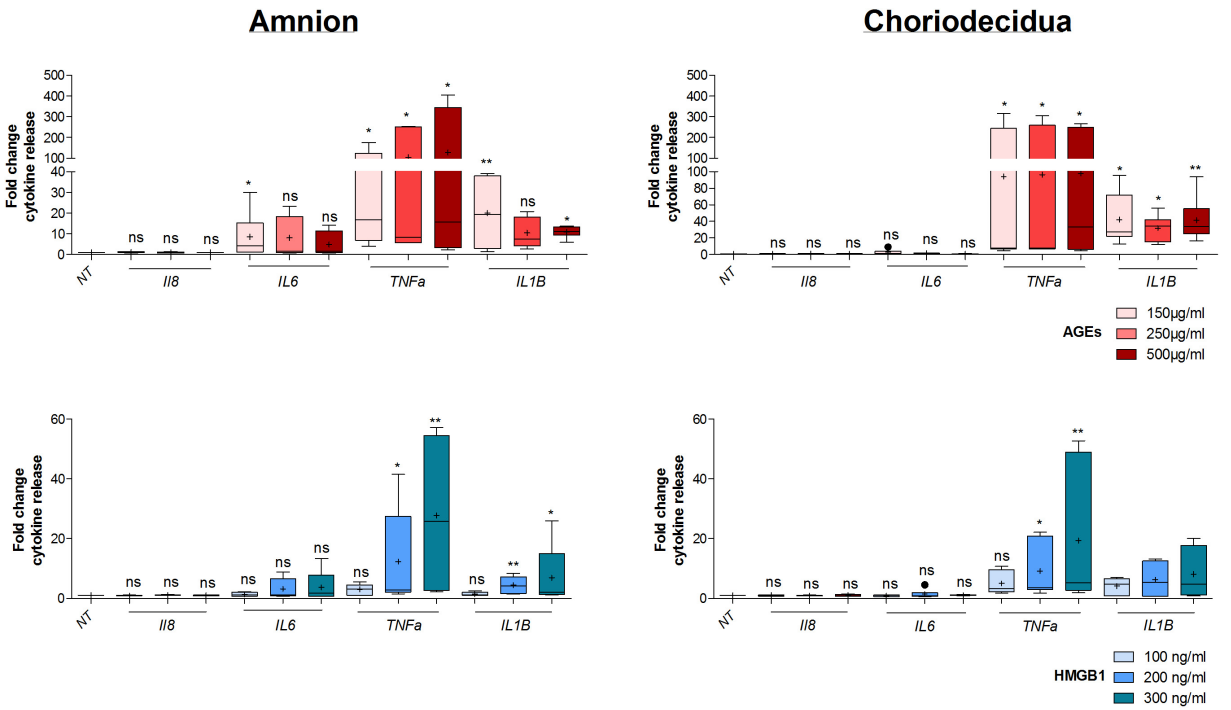

FIGURE 5 | Dose response effect on cytokine release by AGEs and HMGB1 into fetal membrane explants. Evaluation of cytokine release (IL8, IL6, TNFα, IL1β) after 18 h of dose effect treatment of AGEs (150, 250, and 500 µg/ml) and HMGB1 (100, 200, and 300 ng/ml) on the amnion or choriodecidua explants was evaluated by Multiplex assay ($N = 3$ in duplicate). Statistical analysis was performed using a Kruskal–Wallis one-way ANOVA test followed by a Dunn's post-test. * means $p < 0.05$, ** means $p < 0.01$. Results are presented in Tukey boxes, and means are indicated by "+."

(Bredeson et al., 2014; Baumbusch et al., 2016). This could be an exacerbation of inflammatory processes mediated by HMGB1, a major player in labor events (Stephen et al., 2015). In addition, AGEs levels in maternal plasma were described as more important during the first trimester for pregnancies with preterm labor or pPROM (Kansu-Celik et al., 2019). However, there is still a lack of knowledge about which receptor recognizes these alarmins and causes inflammation in the FMs. Some studies have described an overexpression of the RAGE in the placenta and maternal serum in cases of pPROM and also a progressive increase of the soluble isoform of RAGE (sRAGE), acting as a decoy, during pregnancy and then finally decreasing at term (Romero et al., 2008; Yan et al., 2018). Moreover, plasmatic sRAGE levels were found to be lower in patients with pPROM, suggesting an over-activation of the RAGE pathway (Hájek et al., 2008). In this way, our work aimed to enlighten the implication of the RAGE in sterile inflammation in FMs.

The expression of the RAGE in FMs has already been described at term but had not been described during the different trimesters of pregnancy. Presently, we conducted a global exploration of the RAGE axis in both FMs layers (amnion and choriodecidua). First, we proved that FMs not only expressed the RAGE and HMGB1 during all three trimesters of pregnancy, but it also expressed Diaphanous-1 and Myd88, two intracellular adaptors required for inflammatory activity of the RAGE. After that, we observed the presence of the RAGE protein in the amniotic epithelium, the layer directly exposed to AF alarmins and also in all choriodecidua. Thus, we found a differential RNA and protein expression between not only the amnion and choriodecidua and also between the ZAM and the rest of the FMs, the ZIM. Indeed, we showed the overexpression of the RAGE in the amnion compared to the choriodecidua and, above all, in the ZAM compared to the ZIM. These findings strengthen the idea of RAGE participation and importance in the ROM process as previously suggested (Rzepka et al., 2015). Plus, we demonstrated HMGB1 levels to be more important in the amnion. This was not very surprising; indeed, literature already described an increase in sterile inflammation linked with HMGB1 on the fetal side of the FMs, more precisely, in the amnion epithelial cells (Romero et al., 2011). But our work brings the first data on RAGE adaptors in fetal membranes and this is not negligible. In fact, it is currently well known that the RAGE is deficient in intrinsic tyrosine kinase activity and requires intracellular adaptors to induce cell signaling cascades. In this way, a yeast-two-hybrid experiment was achieved and identified a binding partner of RAGE cytosolic domain, the protein Diaphanous-1. Meanwhile, there is no proof that Diaphanous-1 is required for all RAGE induced-transduction cascades; however, some studies reported its implication in protein/signal pathway stimulation triggered by RAGE ligands (Xu et al., 2010; Touré et al., 2012). Hudson et al. (2008) also demonstrated that downregulation of Diaphanous-1 expression by RNA interference inhibited RAGE-mediated activation of Rac-1 and Cdc42 and, in parallel, RAGE ligand-stimulated inflammatory, vascular, and cell migration

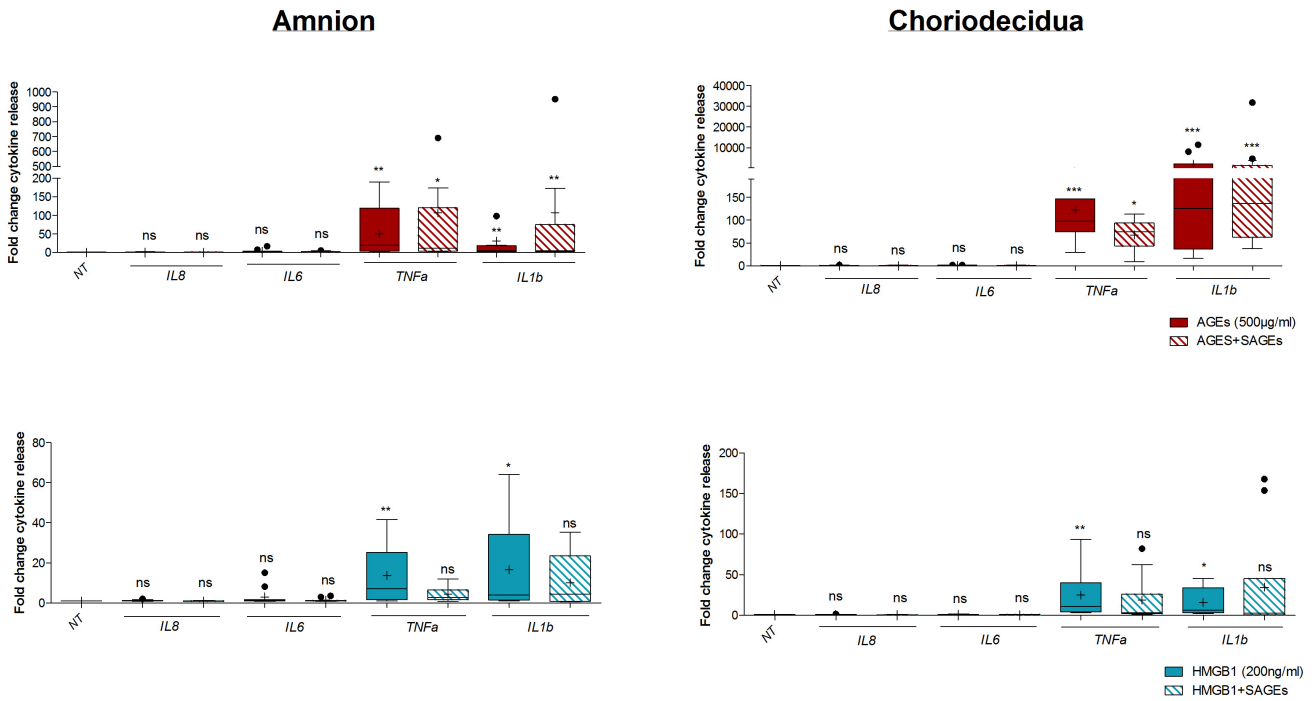

FIGURE 6 | Impact of RAGE inhibition on the induction of inflammatory response by AGEs and HMGB1 alarmins in the amnion and choriodecidua. Pro-inflammatory cytokine (IL8, IL6, TNFα, IL1β) secretion was quantified by Multiplex assay after 18 h of treatment with AGEs (500 µg/ml) or HMGB1 (200 ng/ml) in the presence or absence of RAGE inhibitor, SAGEs (500 µg/ml) ($N = 5$ in duplicate). Each treated condition was reported to corresponding not treated (NT) either without SAGEs or with SAGEs, which were both fixed to one. Statistical analysis was performed using a Kruskal–Wallis one-way ANOVA test followed by a Dunn's post-test. * means $p < 0.05$, ** means $p < 0.01$, *** means $p < 0.001$. Results are presented in Tukey boxes, and means are indicated by "+."

responses. Diaphanous-1 aside, the RAGE owns other adaptor proteins, such as TIRAP or MyD88, shared with the toll-like receptors TLR2 and TLR4. The Sakaguchi group revealed that ligand binding leads to phosphorylation of the RAGE cytoplasmic domain by protein kinase Cζ (pSer391), promoting TIRAP and MyD88 interaction. Furthermore, blocking TIRAP and MyD88 considerably abolished ligand-activated RAGE inflammatory signaling (Akt, p38 MAP kinase, NFκB) (Sakaguchi et al., 2011). In our study, we demonstrated an overexpression of Diaphanous-1 in the amnion, unlike Myd88, which was found to be expressed more in the choriodecidua. These data may suggest a layer-specific signaling couple, RAGE/Diaphanous-1 in the amnion and RAGE/Myd88 in the choriodecidua. Additionally, considering that RAGE and TLR2/4 partly share an intracellular signaling pathway, including MyD88 binding, we could suppose cooperation between RAGE and TLRs in immune response in choriodecidua, explaining why MyD88 was overexpressed in this layer, closer to the genito-urinary microbiota. Indeed, fetal membranes are already known to respond to different types of bacteria by modifications of TLR expression patterns (Abrahams et al., 2013).

As previously stated, AGEs or HMGB1 have been described as inducing cytokine release (IL6, IL8, TNFα, IL1β) in the FMs but without making a distinction between both layers. To investigate a possible differential response to ligands between the amnion and choriodecidua, we decided to perform our treatments with a dissociation of these two sheets using either AGEs or HMGB1. First, we confirmed results from previous studies with TNFα and IL1β release in response to AGEs and HMGB1 in both the amnion and choriodecidua (Lappas et al., 2007; Bredeson et al., 2014; Plazyo et al., 2016). In addition to such results, we did not find the same results for IL8 and IL6 with their release not being stimulated by any dose except for IL6 in the amnion by AGEs at 150 µg/ml. This contrast with previous results can be explained by the use of different concentrations of alarmins. For example, the Lappas group used concentrations of 1 mg/ml of AGEs, and those used for HMGB1 were between 10 ng and 50 µg/ml for Plazyo and colleagues and 1 and 50 ng/ml by the Bredeson team. Then, in our study, for IL6 release, we dissociated layers and could, therefore, hypothesize that only the amnion produces such interleukin in response to AGEs or to HMGB1.

Finally, the major finding of this study was that RAGE inhibition by SAGEs decreased or aborted the TNFα release for both alarmins, indicating that RAGE is required for FMs to initiate this cytokine production. However, contrary to choriodecidua, in amnion, it seems RAGE is not the only actor needed in TNFα release induction because the induction was diminished and not aborted. Globally, this result is of primary importance, as TNFα could activate NFκB complex and the production of the granulocyte macrophage colony-stimulating factor (GM-CSF), which is described to be the critical intermediate for FMs weakening by the intervention of specific

proteases (Kumar et al., 2014). The release of IL1β was only inhibited by SAGEs when tissues were treated by HMGB1 and not by AGEs. Considered together, the results obtained in our work proved for the first time that the RAGE is directly implied in the inflammatory response in human FMs in a ligand and layer-dependent manner.

Our study constitutes the direct evidence of RAGE action in FMs weakening, which is an essential process for the ROM, adding a feature of the pathophysiological partition of the RAGE in the story of childbirth. Further studies are required to elucidate which intracellular pathway, among NFκB and MAPK kinases, for example, leads to this RAGE-dependent cytokine release in FMs, but also which cellular type inside the amnion or choriodecidua has the ability to react to the presence of alarmins.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The Institutional Local Ethics Committee, structure of the University Hospital of Clermont-Ferrand (specialized for Human clinical questions) approved this study and the research protocol. Healthy fetal membranes were collected after receiving oral informed consent (according to the French law named "Huriet-n°88–1138" which considers placenta and fetal membranes as chirurgical wastes) from the patients in the "Centre Hospitalier Universitaire Estaing" (Clermont-Ferrand, France).

AUTHOR CONTRIBUTIONS

HC designed and performed the experiments, and wrote the manuscript. ML and CB helped HC to carry out some experiments. JD helped with Multiplex assays. RM-Q performed lactate dehydrogenase assays. DG allowed HC to obtain human fetal membranes from patients in the Centre Hospitalier Universitaire Estaing (Clermont-Ferrand, France). VS and LB supervised the project.

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REVIEW
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Organ-On-Chip Technology: The Future of Feto-Maternal Interface Research?

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The placenta and fetal membrane act as a protective barrier throughout pregnancy while maintaining communication and nutrient exchange between the baby and the mother. Disruption of this barrier leads to various pregnancy complications, including preterm birth, which can have lasting negative consequences. Thus, understanding the role of the feto-maternal interface during pregnancy and parturition is vital to advancing basic and clinical research in the field of obstetrics. However, human subject studies are inherently difficult, and appropriate animal models are lacking. Due to these challenges, *in vitro* cell culture-based studies are most commonly utilized. However, the structure and functions of conventionally used *in vitro* 2D and 3D models are vastly different from the *in vivo* environment, making it difficult to fully understand the various factors affecting pregnancy as well as pathways and mechanisms contributing to term and preterm births. This limitation also makes it difficult to develop new therapeutics. The emergence of *in vivo*-like *in vitro* models such as organ-on-chip (OOC) platforms can better recapitulate *in vivo* functions and responses and has the potential to move this field forward significantly. OOC technology brings together two distinct fields, microfluidic engineering and cell/tissue biology, through which diverse human organ structures and functionalities can be built into a laboratory model that better mimics functions and responses of *in vivo* tissues and organs. In this review, we first provide an overview of the OOC technology, highlight two major designs commonly used in achieving multi-layer co-cultivation of cells, and introduce recently developed OOC models of the feto-maternal interface. As a vital component of this review, we aim to outline progress on the practicality and effectiveness of feto-maternal interface OOC (FM-OOC) models currently used and the advances they have fostered in obstetrics research. Lastly, we provide a perspective on the future basic research and clinical applications of FM-OOC models, and even those that integrate multiple organ systems into a single OOC system that may recreate intrauterine architecture in its entirety, which will accelerate our understanding of feto-maternal communication, induction of preterm labor, drug or toxicant permeability at this vital interface, and development of new therapeutic strategies.

Keywords: organ-on-a-chip, microfluidic lab-on-a-chip, fetal membrane, amniochorion, extracellular matrix

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INTRODUCTION

The challenges and limitations in studying complex human organ or organ systems have spurred interdisciplinary collaboration to develop advanced human cell culture platforms that better mimic the structure and functions of human organ systems for studying their physiological and pathological processes. The combination of microfabrication, microfluidics, and induced pluripotent stem cell (iPSC) technologies has provided many physiological models that better mimic human anatomy, functions, and responses more accurately as seen *in vivo* than traditional 2D cell culture and some animal models (Liu et al., 2018; Sances et al., 2018; Ramme et al., 2019; Jagadeesan et al., 2020). These platforms, termed organ-on-chips (OOCs) or also called microphysiological systems (MPSs), can provide compartmentalized chambers that enable culturing and organizing cellular, extracellular matrices (ECMs), and other microenvironmental layers within these compartments (Huang et al., 2017; Mondrinos et al., 2017; Pasman et al., 2018), while still providing avenues for cellular signals, and sometimes even cells themselves, to migrate between the compartments through interconnected fluid paths (Ren et al., 2017; Richardson et al., 2019b). These systems allow researchers to test many different biomolecular factors under a more physiologically relevant *in vitro* environment, leading to a better understanding of human physiology through gathering significant amounts of data much faster and potentially much more cost-effectively (Huh, 2015; Maschmeyer et al., 2015; Gori et al., 2016; van der Helm et al., 2016; Bein et al., 2018; Guo et al., 2018; Carvalho et al., 2019). In the United States, significant investments made by the Defense Advanced Research Project Agency (DARPA) and the National Institutes of Health (NIH, especially the National Center for Advancing Translational Sciences) have spurred this area in the past decade. Currently, many pharmaceutical and biotechnology companies, as well as many government entities such as the NIH, the Food and Drug Administration (FDA), and Environmental Protection Agency (EPA) are actively interested in utilizing validated OOC systems to conduct pharmaceutical and chemical toxicity studies as well as collect pre-clinical data due to their ability in better replicating human physiology and responses (Capulli et al., 2014; Esch et al., 2015; Konar et al., 2016; Balijepalli and Sivaramakrishan, 2017).

While the goal of OOC technology is not to build whole living organs, these OOC systems are designed to establish a minimally functional unit of organ systems that can better recapitulate certain aspects of human physiology in *in vitro* model systems. Over the past decade, several studies have ushered in the era of OOC technology by replicating organs such as the heart (Zhang et al., 2015, 2016; Jastrzebska et al., 2016; Wan et al., 2018), lung (Huh, 2015; Konar et al., 2016; Shrestha et al., 2020), intestine (Kim et al., 2012; Bein et al., 2018; Guo et al., 2018), liver (Maschmeyer et al., 2015; Esch et al., 2016; Gori et al., 2016; Ramme et al., 2019), kidney (Maschmeyer et al., 2015; Wilmer et al., 2016; Ashammakhi et al., 2018; Ramme et al., 2019), skin (Maschmeyer et al., 2015; Materne et al., 2015; Mori et al., 2017; van den Broek et al., 2017; Bal-Ozturk et al., 2018), blood–brain barrier (BBB) (van der Helm et al., 2016; Jeong et al., 2018; Jagadeesan et al., 2020), bone (Hao et al., 2018; Truesdell et al., 2020), eye (Dodson et al., 2015; Bennet et al., 2018; Haderspeck et al., 2019), and ovary (Nagashima et al., 2018; Weng et al., 2018), to name a few. For a more thorough review of currently available OOCs, refer to these reviews (An et al., 2015; Esch et al., 2015; Balijepalli and Sivaramakrishan, 2017; Low and Tagle, 2017; Kimura et al., 2018). Although they each started with simplistic models, each of these platforms has now been advanced to adapt novel physiologically relevant functions such as cellular contractions (i.e., heart, lung, and eye) (Huh, 2015; Qian et al., 2017; Seo et al., 2019), drug synthesis and excretion (i.e., liver and kidney) (Paoli and Samitier, 2016; Deng et al., 2019), barrier functions (i.e., skin and brain) (Jeong et al., 2018; Mieremet et al., 2019), dynamic flow of blood, air, or fluid interfaces (i.e., heart, lung) (Ribas et al., 2016; Artzy-Schnirman et al., 2019), and even co-culture with bacterial microbiomes (i.e., intestine) (Jalili-Firoozinezhad et al., 2019) in order to replicate the human organ systems of interest. In addition, multiple organ chips can be integrated, either physically through tubing or microfluidic channels or virtually by sending effluents from one OOC to another OOC, to create *in vitro* models of interconnected organ systems, with the ultimate goal of mimicking the entire human physiology (Maschmeyer et al., 2015; Materne et al., 2015; Kimura et al., 2018; Ramme et al., 2019).

From a basic science perspective, microfabricated microfluidic OOC platforms that replicate the microarchitecture of complex organ systems have opened up new experimental procedures to researchers that can utilize such platforms to study contributions of individual cells, cell–cell and cell–ECM interactions, and various biochemical factors to normal organ functions, and also how such functions are influenced by various factors that can be experimentally applied. Furthermore, these models can be extended to mimic a pathologic state, study disease physiology, and mechanisms of action, highlighting the usefulness of these devices in advancing our understanding of human physiology. The recent investments made by the US NIH (NCATS and many other NIH institutes) focusing on the development and utilization of disease OOCs are expected to advance this field further (Ronaldson-Bouchard and Vunjak-Novakovic, 2018; Ouchi et al., 2019; Park et al., 2019; Taylor et al., 2019; Vatine et al., 2019; Wang et al., 2019; Zhao et al., 2019).

From a clinical perspective, *in vitro* cell culture techniques and *in vivo*, small and large animal models, have been the backbone to collect pre-clinical data (Umscheid et al., 2011). The ever-increasing cost of new drug development, stemming in large part due to the large number of drugs that fail at the clinical trial phases due to toxicity or lack of efficacy, or which show conflicting results in animal models, have led to researchers beginning to look for methods that can better predict the toxicity and efficacy of potential drug compounds. OOCs are poised to fill this gap by providing physiologically relevant platforms for better modeling health and disease states of human organ systems. Currently, a variety of OOC platforms are being used in these settings, to model processes such as: (1) mode and mechanism of action, (2) pharmacokinetics and pharmacodynamics, (3) toxicity, (4) efficacy, and (5) dose–response (Luni et al., 2014; Abaci and Shuler, 2015; Esch et al., 2015; Ribas et al., 2016; Wilmer et al., 2016; Balijepalli and Sivaramakrishan, 2017;

Low and Tagle, 2017; Bal-Ozturk et al., 2018; Jodat et al., 2018; Kimura et al., 2018; Artzy-Schnirman et al., 2019; Haderspeck et al., 2019; Mittal et al., 2019; Pemathilaka et al., 2019b; van den Berg et al., 2019).

Although many fields have seen the development and advancement of OOC platforms to model physiological and pathological states of their organ systems of interest, the area of obstetrics is only now applying this emerging technique to study pregnancy and preterm birth (Blundell et al., 2016, 2018; Lee et al., 2016; Gnecco et al., 2017; Pemathilaka et al., 2019a,b; Richardson et al., 2019a; Yin et al., 2019). Unlike other single organ model systems, pregnancy introduces new fetal-derived organs within the mother's uterine cavity (i.e., placenta, umbilical cord, fetus, and fetal membranes) for a period of 9 months (**Figure 1**) (Menon et al., 2016; Richardson et al., 2018b). These new organs play an essential role in pregnancy maintenance, development, and induction of parturition. Two of these fetal-derived organs, namely, the placenta (**Figures 2A,B**; top dotted box) and fetal membrane (also known as amniochorion membrane or placenta membrane) (**Figures 2B,C**; bottom dotted box), create the feto-maternal interface throughout gestation; (1) between placenta and decidua basalis and (2) between fetal membranes and decidua parietalis. The decidua basalis is where implantation takes place and the basal plate is formed. This can be subdivided into a zona compacta and a zona spongiosa, where the detachment of the placenta takes place following birth. The decidua capsularis lies like a capsule around the chorion, while the decidua parietalis remains on the opposite uterus wall.

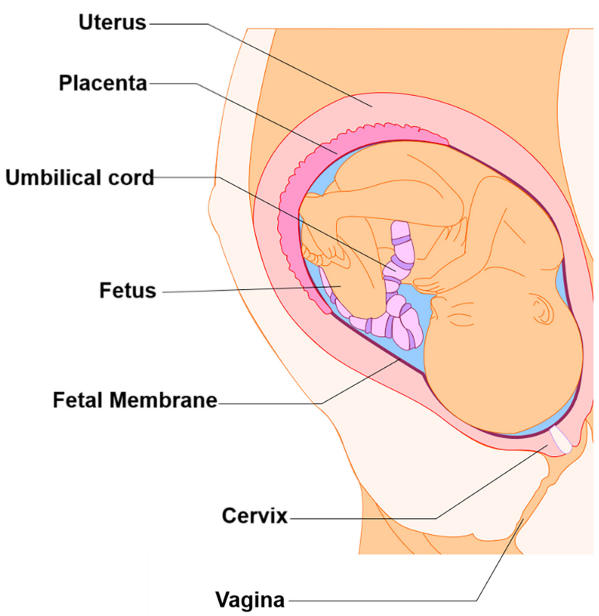

FIGURE 1 | Intra-uterine tissue anatomy. An illustration of the anatomy of the intra-uterine tissue broken down into maternal and fetal components. Maternal tissues comprise of the uterus (i.e., Myometrium), cervix, and vagina, while the fetal tissues include the placenta, umbilical cord, fetus, and fetal membranes.

Around the fourth month of gestation, the fetus is so large that the decidua capsularis comes into contact with the decidua parietalis. The merging of these two deciduae causes the uterine cavity to obliterate and forms the two feto-maternal interfaces. The placenta is comprised of the decidua basalis connected to the myometrium (gray), tertiary chorionic villi and intervillous space (light purple), and the reflective amniochorion membrane (blue and yellow cells). The placenta plays a critical role in maintaining pregnancy by regulating maternal metabolism, endocrine and immune functions in addition to providing blood flow [arteries (blue) and veins (red)], nutrients, and oxygen to the fetus, while removing waste products such as carbon dioxide (Jabareen et al., 2009; Mauri et al., 2013; Edey et al., 2018) (**Figures 2A,B**). The fetal membrane, which surrounds the baby throughout gestation, provides essential immune, endocrine, and mechanical functions (**Figures 2B,C**) that maintain pregnancy (Jabareen et al., 2009; Boldenow et al., 2013; Mauri et al., 2013, 2015; Perrini et al., 2015; Menon, 2016; Menon et al., 2016, 2017; Sato et al., 2016; Edey et al., 2018; Shah et al., 2019). They are comprised of two epithelial layers, the amnion (blue) and chorion (yellow), separated by an ECM containing mesenchymal cells (purple). The chorion layers are connected to the first layer of the decidua, termed the parietalis (green). As reviewed by Menon and Moore recently, this is one of the least studied intrauterine organs as it is often considered as an extension of the placenta or a dead tissue upon delivery (Menon et al., 2016). At term or preterm, redox imbalances within the intrauterine cavity induce a telomer-dependent, p38MAPK-mediated, cellular senescence in the amnion epithelial cells (AECs) (**Figure 2C**), which propagate damage-associated molecular patterns (DAMPs) and senescence-associated secretory phenotypes (SASPs) to the maternal decidua (**Figure 2C**), contributing to the initiation of labor (Menon et al., 2013, 2016; Behnia et al., 2015; Polettini et al., 2015; Sheller et al., 2016; Dixon et al., 2017; Hadley et al., 2018). Fetal membrane-derived signals are one of the essential fetal-derived messages of parturition at term and preterm (Menon et al., 2018; Menon, 2019). In this review, we will focus on the fetal membranes by (1) highlighting the conventionally utilized techniques to study their physiology and contribution to parturition, (2) discuss major design elements of OOCs that enable multi-layer co-cultivation of cells, (3) introduce recently developed feto-maternal interface OOC (FM-OOC) models, and (4) provide a perspective on the future development and impact of pregnancy-related OOCs in the field of obstetrics.

CURRENT METHODS TO STUDY THE FETO-MATERNAL INTERFACES AND THEIR LIMITATIONS

Fetal membranes (structure detailed in **Figure 2C**) are different from the placenta in terms of their origin, structure, cell types, and functions (Gude et al., 2004; Menon et al., 2018; Richardson et al., 2018b). Not surprisingly, due to these differences, the *in vivo* and *in vitro* models used to study these two distinct feto-maternal interfaces are also unique. Below we will discuss: (1) the similarities and differences between human anatomy

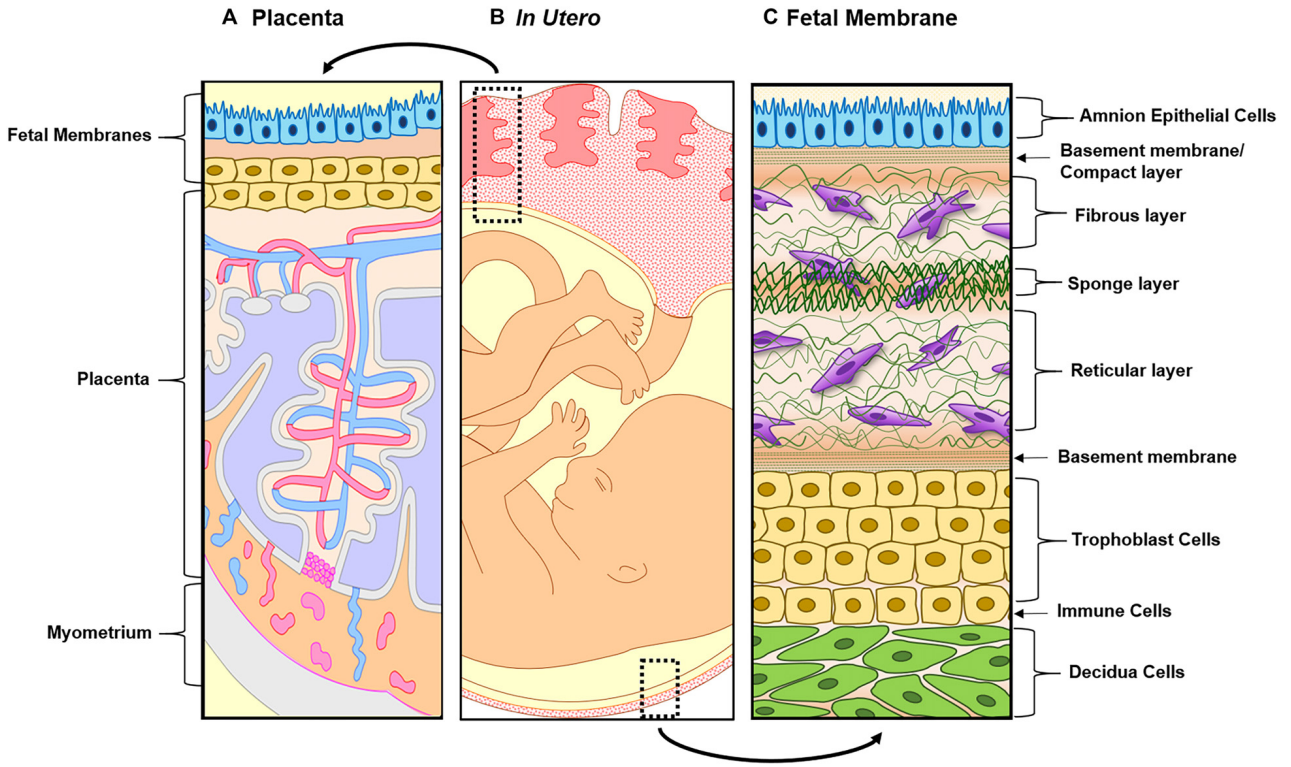

FIGURE 2 | Illustration of both feto-maternal interfaces *in utero*. **(A)** The left side represents the placenta, the site of nutrients, oxygen, and waste exchange for the growing fetus. The placenta is attached to the maternal side by the decidua basalis next to myometrium (gray) and the fetal side through the fetal membranes (amnion in blue and chorion in yellow). This image highlights the tertiary chorionic villi and arteries (red) and veins (blue), respectively. **(B)** Overview of the two feto-maternal interfaces in relation to the fetus. The top box outlines the cross-section of the placenta and the bottom box outlines the cross-section of the fetal membrane. **(C)** The description starts from the innermost layer (amnion) and ends at the maternal decidua. Amnion epithelial cells (blue) are connected to the first layer of the ECM called the basement membrane/compact layer (green strips). The fibroblast (top), spongy (middle), and reticular layers (bottom) follow, containing amnion and chorion mesenchymal cells (purple). The chorion (yellow) is connected to the ECM through a basement membrane (green stripes and is made up of two types of cells: chorion laeve cells and chorion trophoblast cells. The chorion interfaces with the maternal decidua (green), connecting the fetal layers to the maternal compartments of the uterus.

and commonly used large and small animal models and (2) the advantages and limitations to current *in vitro* and *ex vivo* techniques.

Limitations of Animal Models and Current *in vitro* and *ex vivo* Culture Techniques

Animal Models

Large and small animal models [i.e., non-human primates (NHP) and mice] are often used for fetal membrane studies. However, differences in pregnancy physiology and structure and uterine environment and cost to conduct studies often hamper the use of these models. NHP models most closely resemble the human fetal–maternal interface (**Figure 3A**), only deviation being the addition of densely packed fibrous layer covering the basal side of the amnion epithelium termed "microfibrils" (**Figure 3B**; dark green). These microfibrils could hinder communication between the AEC and ECM layers, which in humans have been shown to be vital for pregnancy maintenance and labor signaling at term. Apart from this, NHPs are anatomically and functionally the most similar to humans, and serve as a reproducible model that enables longitudinal testing of experimental outcomes (**Table 1**). However, the limitations of this large animal model lie in: (1) the cost of each animal and future housing expenses, (2) handling difficulties, and (3) the need for a proper facility to conduct experiments (**Table 1**). While NHPs are the gold standard for large animal cytotoxicity studies, murine models such a CD1 and C57BL/6 mice are commonly utilized for small animal pre-clinical experiments. This is primarily due to the fact that murine experiments are easy to conduct, cost-effective, and have a short gestation (**Table 1**). Although valuable information can be gained from such models, the vast differences in anatomy (i.e., vasculature and maternal layers), and the induction of parturition (i.e., luteolysis), limit their use (**Table 1**). Regarding fetal–maternal interface anatomy, during murine gestation, the "amniotic sac" develops and surrounds each fetus, mimicking the fetal membrane. The amniotic sac is comprised of two epithelial layers; an amnion epithelial monolayer (blue in **Figure 3C**) and a multilayer chorion trophoblast cells (yellow in **Figure 3C**). Between these layers, loose collagen fibers support mesenchymal cells (purple in **Figure 3C**) and maternal blood vessels in the

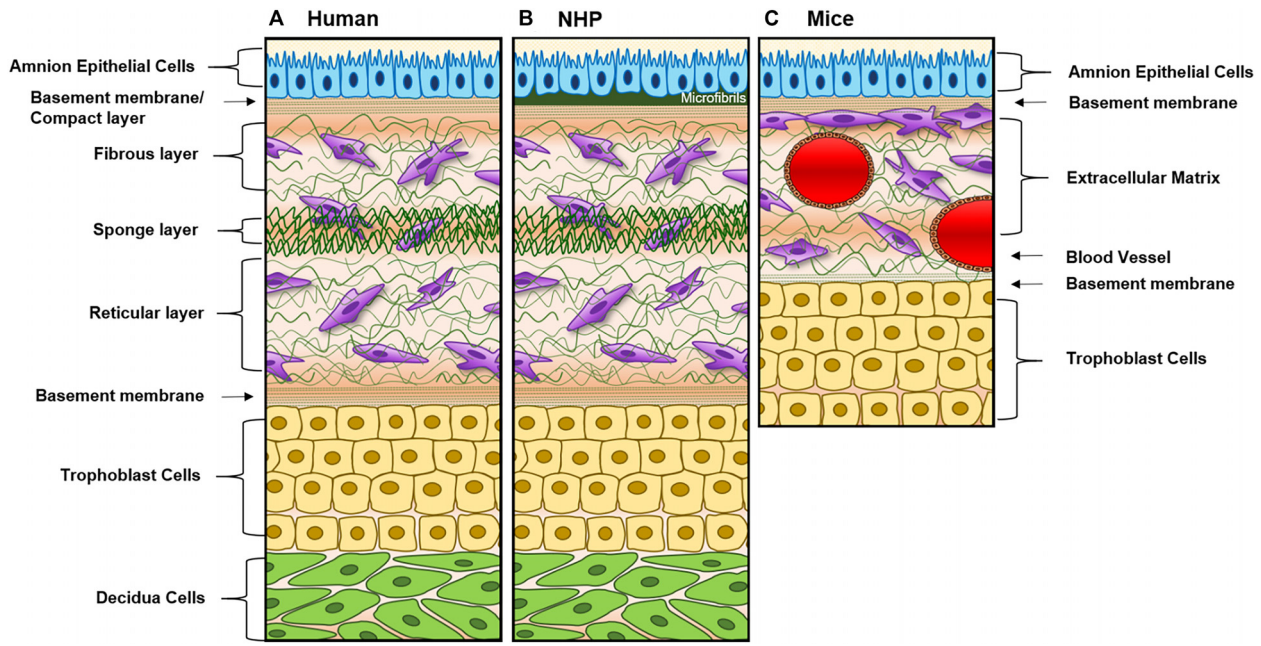

FIGURE 3 | Diagram of fetal membranes anatomical differences between species. **(A)** Illustration representing the human fetal membrane. The human fetal membrane, or feto-maternal interface, starts with the innermost layer (amnion) facing the amniotic cavity and ends with the maternal decidua. Within the intrauterine cavity, the amnion epithelial cells (AECs) connected to the basement membrane (green stripes) are bathed in amniotic fluid (yellow) and comprise the first layer. Below AECs, the ECM is comprised of compact, fibrous, spongy, and reticular layers, all containing mesenchymal cells derived from the amnion and chorion (purple). Chorion trophoblast cells (CT; yellow) are attached to the ECM via another layer of the basement membrane on its apical side and to the maternal decidua (green) on its basal side. **(B)** Schematic of non-human primate (NHP) fetal membranes, currently the best animal model used in the field. It is almost identical to human fetal membranes; however, specific to NHPs, the AEC layer interfaces with a thick, fibrous, collagen layer termed "microfibers" (Owiti et al., 1989) before the basement membrane/compact layer of the ECM. **(C)** The amniotic sac of a mouse is comprised of two epithelial layers; amnion epithelial monolayer (blue) and a multilayer of chorion trophoblast cells (yellow). Between these layers, loose collagen fibers support mesenchymal cells (purple) and maternal blood vessels in the ECM. This tissue does not contain a maternal interface (i.e., decidua) as other mammalian models.

ECM (**Figure 3C**). This tissue does not contain a maternal interface (i.e., decidua), other than maternal blood flow, like other mammalian models. These anatomical differences are the biggest hindrance when conducting physiological or pharmaceutical related experiments in murine systems.

A full list of the advantages and limitations of each model described in this section can be found in **Table 1**, and the anatomical differences can be seen in **Figure 3**.

Cell Sources for *in vitro* Models

The standard in fetal membrane research is maintaining membranes as explants *in vitro* (Fortunato et al., 1994) or culturing primary cells (Menon et al., 2013; Sheller et al., 2016; Hadley et al., 2018; Jin et al., 2018; Richardson et al., 2020b), both obtained from discarded human fetal membranes dissected after placental delivery. Though this brings in patient-to-patient variability (**Table 1**), these approaches maintain some of the *in vivo* characteristics such as cytoskeletal organization (Menon et al., 2018; Richardson et al., 2018b, 2020b), endocrine and paracrine signaling (Myatt and Sun, 2010; Behnia et al., 2015), inflammatory responses (Menon et al., 2009; Noda-Nicolau et al., 2016), as well as immune regulatory factors (Fortunato et al., 1998, 2001). Protocols documenting amnion (i.e., AEC and AMC) cell isolation techniques are well established (Kendal-Wright, 2007; Menon et al., 2013; Sato et al., 2016; Jin et al., 2018). However, although it is not impossible to isolate and culture primary chorion mesenchymal and trophoblast cells (CMC and CT) [99, 108], due to many *in vitro* challenges (isolation, culture conditions, passage-related issues, and transition properties), researchers have turned to use immortalized placenta-based trophoblast cells (i.e., BEWO and JEG-3) derived from carcinomas to replicate this layer [109]. As fetal membrane CTs reside in a functionally different region and perform distinct functions than placental trophoblast, the use of placental trophoblasts-derived cell lines to study chorionic membrane trophoblast functions are not ideal. The various cell types and cell sources that could be utilized to study the fetal membranes and feto-maternal interface are summarized in **Table 1**, together with their advantages and limitations.

In vitro Cell Culture Techniques

Two-dimensional (2D) single cell type culture experiments are most easy to run and low cost, and thus broadly utilized (Kendal-Wright, 2007; Menon et al., 2013; Meng et al., 2016; Sato et al., 2016; Feng et al., 2018b; Hadley et al., 2018; Jin et al., 2018). However, regardless of the cell origin (i.e., primary or immortalized) or cell layer (i.e., amnion or chorion), the major drawback is the limitations stemming from studying only a small

part of the whole organ. Co-culturing two or more cell types allow researchers to study the organ system in a more holistic way, including studying cell–cell and cell–ECM interactions. Transwell co-culture is the current standard protocol to represent the amnion (i.e., AEC and AMC), amniochorion (i.e., AEC and CT or BEWO), and the feto-maternal interface (i.e., AEC and decidua) (Blanco et al., 2009; Talayev et al., 2010; Magatti et al., 2015; Wu et al., 2017; Lee et al., 2018; Richardson et al., 2019a). Despite providing a much more physiologically relevant model compared to 2D mono- or mixed culture, transwell culture has a variety of limitations, as summarized in **Table 1**.

Ex vivo Tissue Culture Techniques

Culturing fetal membrane tissues or biopsy-based explants obtained from discarded human fetal membrane from scheduled cesarean deliveries are commonly utilized for tissue-level culture and studies (Fortunato et al., 1994; Menon et al., 2011; Menon et al., 2014; Richardson et al., 2017a; Ayad et al., 2018). Additionally, live fetal membrane samples can be mounted into imaging chambers allowing for cellular and collagen visualization over time. Along with traditional explant treatments, these studies mimic biomechanical stressors, such a stretch, in order to delineate membrane weakening leading to rupture. These types of experiments enable a variety of molecular and biochemical assays using a more physiologically relevant model system and thus have significantly contributed to our current knowledge of fetal membrane physiology in the field (Fortunato et al., 1994; Miller and Loch-Caruso, 2010; Uchide et al., 2012; Boldenow et al., 2013; Menon et al., 2014; Kumar et al., 2016; Martin et al., 2017; Feng et al., 2018a; Hung et al., 2019). While explant culture maintains many advantages compared to other *in vitro* techniques, there are also many limitations, as summarized in **Table 1**.

CO-CULTURE ORGAN-ON-CHIP DESIGNS AND FUNCTIONS

Most organ systems are composed of two or more cell types that are arranged in a specific way to create various microarchitectures, where these multiple cell types closely interact and function together, giving rise to the unique structure and functions of each organ system (Bhatia and Ingber, 2014; An et al., 2015; Huh, 2015; van der Helm et al., 2016; Wilmer et al., 2016; Bein et al., 2018; Jodat et al., 2018; Sances et al., 2018). To recapitulate such complex multi-cellular structures, the majority of OOC systems (also called tissue chips or MPSs) require two or more different cell types to be co-cultured in specific arrangements. In addition, various interstitial flow (Artzy-Schnirman et al., 2019) and blood flow also directly access these multi-cellular architectures in a specific way (Ribas et al., 2016); thus, the multiple cell culture compartments of OOCs also need to be accessed by various fluids, creating distinct microenvironments for each cell types and cellular layers. In addition to cells, various ECMs secreted by cells are also significant components of most organ systems (Mondrinos et al., 2017; Pasman et al., 2018). Thus, OOCs have also to consider incorporating physiologically relevant ECMs. For all OOC systems, it is also essential to be able to monitor the cells and their microenvironment. Thus, being compatible with microscopy is critical. In addition, easy fluidic access to each cell layer, for both applying various biochemical stimuli and being able to analyze secreted metabolites, are also necessary. Here, we first provide a review of typical co-culture OOC architectures, and ECMs used, followed by examples of currently available OOC systems representing the feto-maternal interface.

Co-culture OOC Architectures

Most co-culture OOCs fall under two design categories, "vertical" or "planar" co-culture designs, termed based on the orientation of the multiple microfluidic culture chambers that comprises the OOC (**Figure 4**). Each design has unique advantages and disadvantages, which are described below.

Multi-layered vertical co-culture OOCs are designed to contain vertically stacked cell culture chambers separated by a porous membrane (**Figure 4A**). Here, a semipermeable membrane separates the two vertically positioned cell culture chambers (**Figure 4A**; black arrow), allowing cells to be confined within each cell culture chamber while allowing biochemicals to flow through the membrane freely. This membrane often mimics the basement membrane layer and collagen, thus enabling cell–cell and cell–collagen interactions in the OOC environment (Pasman et al., 2018). The most commonly utilized membranes are the commercially available track-etched polyethylene terephthalate (PET) membrane, the same membrane utilized in transwell culture. These membranes come in varieties of different pore sizes, which can be selected to control the permeability between the cell culture compartments and can also mimic *in vivo* collagen density (i.e., pore size). These membranes can be coated with various ECMs collagens, partially recreating the cell–ECM interface. The two cell types can also be cultured on both sides of this membrane, minimizing the distances between the two cell types and allow better cell-cell interactions. Since these membranes are typically around 10 μm thick (Pasman et al., 2018) and made of plastic, and thus more rigid and thicker than what may be seen *in vivo* (i.e., 2 kPa and 13.4 ± 2.42 μm thick) (Halfter et al., 2013; Richardson et al., 2017b), custom membranes that are more thinner than commercially available ones have also been developed for OOC applications (Sip and Folch, 2014; Mondrinos et al., 2017; Pasman et al., 2018). The vertically positioned co-culture chambers can be fabricated with various materials (i.e., glass, polycarbonate, polyurethanes) and by multiple microfabrication processes (i.e., soft-lithography, laser engraving, CAD-based machining, and 3D printing), providing flexibility for various OOC applications. Overall, the vertical co-culture OOC design structurally mimics the *in vivo* structure, which is one of the major advantages of this design. Several OOC devices mimicking organs such as the lung, gut, and BBB utilize this design (**Table 2**) (Kim et al., 2012; Esch et al., 2016; Jeong et al., 2018).

Despite these features and advantages, two significant limitations of this design are: (1) imaging of each cell culture compartment is relatively difficult due to the difficulty of imaging through the membrane that prevents imaging both cell types and (2) due to its multilayer design, microfabrication steps are more

TABLE 1 | Benefits and limitation of *in vivo* and *in vitro* fetal membrane methodology.

	Model		Sub-type	Benefits	Limitations
In vivo	Murine			• Cost-effective • Easy to handle • Longitudinal sample available	• Anatomical differences • Parturition initiation differences • Small sample size per pup • Large pup number • Endocrine differences
	Non-human primate			• Anatomical similarities • Parturition initiation similarities • Longitudinal sample availability • Reliable large animal model • Small fetal number	• Not cost-effective • Expensive to maintain Hard to handle
	Human			• Preferred primary source • Correct anatomy • Parturition initiation standard	• Clinical trial difficult • Hard to acquire samples • Require infrastructure to store samples properly • Difficult to collect longitudinal samples
	2D cell culture	Sing cell type culture	Primary cells	• Human cells • Physiologically relevant • Maintain *in vivo* characteristics	• Patient-to-patient variability • Difficult to culture • Only studying a part of the whole tissue
In vitro	2D cell culture Transwell co-culture	Sing cell type culture Multi-cell type mixed culture	Immortalized human cells	• Derived from human cells • Sustainable in culture	• Have to prove physiological relevance • *n vivo* characteristics lost • Only studying a part of the whole tissue
			iPSC	• Sustainable in culture • Universal lab standard	• Many cell types do not yet exist • Have not been optimized for the feto-maternal interface • Only studying a part of the whole tissue
				• Ability to study cell–cell interactions	• All cell types are mixed together • Hard to determine signal initiation
		Co-culture		• Ability to study cell–cell or cell–collagen interactions • Ability to study signaling propagation and barrier function	• Difficult to culture cells on both sides the membrane • Low throughput • High signal-to-noise ratio • Cell type limitations • Difficult to expand beyond two cell type co-culture
	3D cell culture	Spheroids		• 3D growths of cells • Better maintain *in vivo* characteristics • High throughput • Mixed co-culture possible	• Small cell number leads to limited phenotypic assays • Time-consuming to form • Not uniform • May not organize properly into proper organ structure
	3D cell culture Whole tissue	Cell sheets		• Full amnion layer • Can be cultured longer than amnion explants Easy to image	• Very fragile • Only mimic the amnion layer • Lacks uniformity •
		3D cell printing		• Can recreate multiple feto-maternal interface layers • Proper tissue organization • Uniform production	• Can apply unwanted shear stress to cells during printing • Time-consuming to characterize for each cell type and ECMs to be printed Have not been demonstrated for feto-maternal interface yet
		Explant culture		• Correct tissue organization • Mimics *in vivo* signaling	• Hard to acquire samples • Require infrastructure to store samples properly • Only culturable for up to 72 h or less

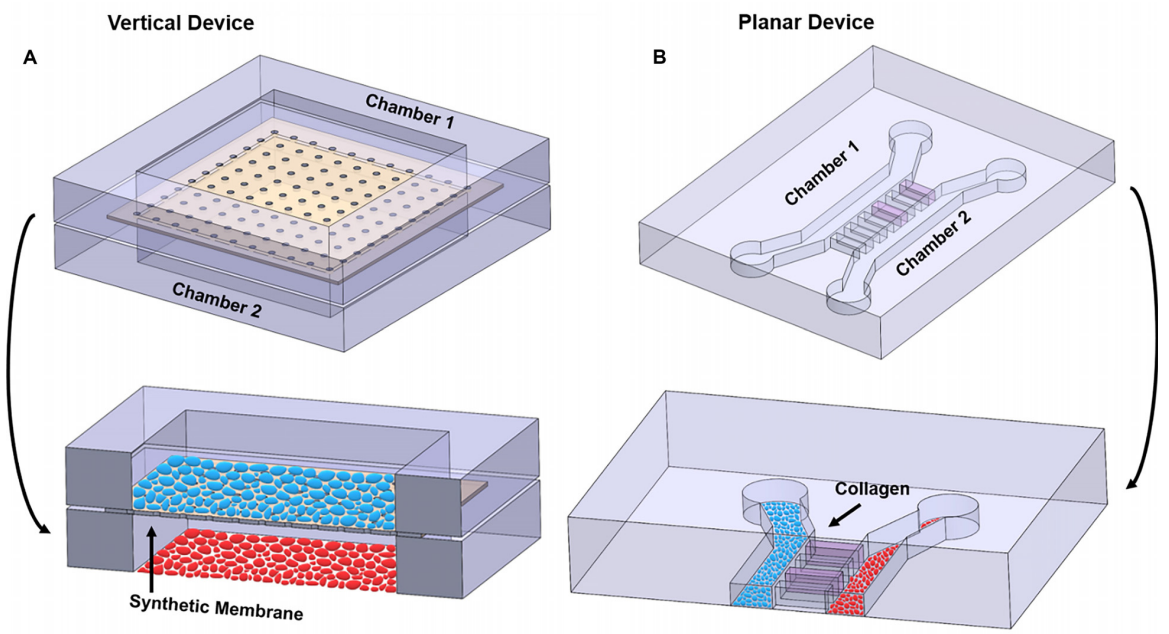

FIGURE 4 | Illustration highlighting the differences between vertical and planar co-culture OOC designs. **(A)** 3D view of two cell culture chambers, stacked on top of each other, to form a "vertical co-culture" OOC device. These two chambers are separated by a semipermeable synthetic membrane (tan structure; black arrow), which contains small pores for cell migration and signal propagation, but too small for cells to freely move between the layers. Blue ellipses are grown on top of the membrane, while red ellipses are grown on the bottom glass substrate. Red and blue ellipses represent two distinct cell populations. **(B)** 3D view of two cell culture chambers aligned next two each other and separated by a set of microchannel arrays to form a "planar co-culture" OOC device. These two chambers are separated by microchannels that can be filled with collagen (pink; black arrow), providing an actual cell–collagen interface. Both blue and red ellipses are grown the bottom glass substrate but in separate chambers. Red and blue ellipses represent two distinct cell populations. The sizes of the microchannels are small enough to prevent cells from freely moving between the culture compartments but large enough for actively migrating cells and biochemicals to move between the compartments. Alternatively, these microchannel arrays can be replaced with a porous gel barrier.

complicated and also reliable sealing of this sandwich structure is challenging. This also means that mimicking any organ structure that is composed of three or more cell layers, which is the case of the fetal membrane and feto-maternal interface, requires assembling multiple such sandwich structures, which pose even more challenges. This could significantly limit its applicability in many complex organ systems. In contrast, a planar co-culture design is ideal for cell visualization and fluid control because all the layers are on the same plane.

Planar co-culture OOCs are designed to contain parallel cell culture chambers separated by porous gel or microchannel arrays (Huh et al., 2011; Gumuscu et al., 2017; Richardson et al., 2019b), all in the same plane (**Figure 4B**). Here, the gel or microchannel array functions as a porous barrier that keeps cells within each cell culture chamber, while allowing various biochemicals to diffuse through. In the case of gel barrier-based systems, gel guiding microstructures (e.g., micropillar array or micro steps) are utilized so that the gel barrier fills only the space between the two culture chambers and prevents the gels that are being loaded to spill over and into the culture chambers (Vickerman et al., 2008; Chung et al., 2009; Funamoto et al., 2012; Osaki et al., 2020; Poussin et al., 2020). Here, the type of gel utilized, and its porosity determine how easily biochemicals can diffuse between the culture compartments. Another design strategy uses arrays of microfluidic channels that are small enough to prevent cells from moving from one compartment to the other (however, still allowing cell migration), but large enough for biochemicals to diffuse through. In this design, the length, size, and number of microfluidic channels control the degree of diffusion. These microfluidic channels can also be filled with various ECM components. In both cases, this gel barrier or microfluidic channel filled with ECM mimics a basement membrane and collagen layer that cells can actively degrade as seen *in vivo*, thus enabling cell–cell and cell–collagen interactions in the OOC environment (Richardson et al., 2019b; Osaki et al., 2020; Poussin et al., 2020). A major advantage of these planar designs is its compatibility and ease in microscopic imaging, as all structures are in the same focal plane and transparent. This makes it also ideal for identifying and monitoring cell–cell communication under different physiological environments, which is often challenging in vertical designs. The planar microfluidic designs are most commonly made out of polydimethylsiloxane (PDMS), but various other plastic that can minimize molecular adsorption can also be utilized (Berthier et al., 2012; Wang et al., 2012). These designs have been used extensively in OOC devices, such as those that mimic organs like the gut, liver, and multiorgan systems (**Table 2**) (Maschmeyer et al., 2015; Gori et al., 2016; Guo et al., 2018; Carvalho et al., 2019; Yin et al., 2019). Limitations of these planar designs also exist, such as difficulty in recreating tight junction barrier formation such as the BBB, and the larger

TABLE 2 | Examples of current co-culture OOC models that utilize vertical or planar co-culture designs.

Lab group	Name of chip	Structure	Functionality	Cell types	Number of chambers	Journal name and year
Maschmeyer et al.	Microfluidic four-organ chip	Planar	Human intestine, liver, skin and kidney co-culture to test drug metabolism	Human HepaRG cell line	6	Lab on a Chip, 2015
				Human primary hepatic stellate cell (HHSteC)		
				Human proximal tubule cell line (RPTEC/TERT-1)		
				Reconstructed human small intestinal barrier		
				Human juvenile prepuce		
Carvalho et al.	Colorectal tumor-on-a-chip	Planar	Precision nanomedicine delivery to a colorectal tumor	HCT-116 cancer cell	2	Science Advances, 2019
				Human colonic microvascular endothelial cell (HCoMEC)		
Y. Guo et al.	Biomimetic gut-on-a-chip	Planar	Drug metabolism in the intestine	Caco-2 cell	Four replicate chambers	Artificial Organs, 2018
Gori et al.	Non-alcoholic fatty liver disease-on-a-chip	Planar	Mimicking non-alcoholic fatty liver disease to understand how it can lead to hepatocellular carcinoma	Human hepatoma HepG2/C3A cell	2	PLOS One, 2016
F. Yin et al.	3D human placenta-on-a-chip	Planar	Establishment of 3D placental barrier and placental response to nanoparticle exposure *in vitro*	BeWo cell		Toxicology *in Vitro*, 2019
				Human choriocarcinoma cell	2	
				Human umbilical vein endothelial cell (HUVEC)		
Jeong et al.	3D blood–brain barrier model	Vertical	Blood–brain barrier function and permeability measurements	Primary astrocyte cell	2	IEEE Transactions on Medical Engineering, 2018
				Mouse brain endothelial cell (C57BL/6)		
Esch et al.	Modular body-on-a-chip	Vertical	Model drug metabolism in the GI tract epithelium and 3D primary liver tissue	Caco-2 Cell		Lab on a Chip, 2016
				Non-parenchymal cell (NPC)	2	
Kim et al.	Human gut-on-a-chip	Vertical	Mimic the flow and microbial flora environment of the gut	Caco-2 Cell	2	Lab on a Chip, 2012
				Human Caco-2 intestinal epithelial cell (Caco-2BBE)		
Huh et al.	Lung-on-a-chip	Vertical	Recreate the lung and test biological function	Human alveolar epithelial cell	2	Science, 2010
				Microvascular endothelial cell		

distances between the co-culture compartments compared to the vertical co-culture OOC designs.

As summarized here, both co-culture OOC designs have been extensively utilized, with no one system being the perfect design, both having several advantages and disadvantages. This means that each OOC system design, even if mimicking the same organ system, must be decided based on what type of experiments researchers need to run and what kind of measurements are needed for such experiments. Understanding the "fit for purpose" concept when designing any OOC system becomes critical, as no one system can completely mimic the complex human organ system. In addition to these two designs, bioprinting of cells or scaffolds can also be utilized to create co-culture OOC devices (Miri et al., 2019; Mittal et al., 2019), but no such OOC devices of fetal membrane and feto-maternal interface exist as of yet, thus are not included in this review.

In vitro Extracellular Matrices

In vertical co-culture OOC designs, synthetic membranes (e.g., track-etched PET membrane) are widely used to mimic the basement membrane (Sip and Folch, 2014; Mondrinos et al., 2017; Pasman et al., 2018) and regulate the communication between the two cell culture compartments. However, such synthetic membranes are quite different from the *in vivo* basement membrane, which is a key biological factor and plays many roles (Guller et al., 1995; Bryant-Greenwood, 1998; Strauss,

2013; Richardson et al., 2017a,b). Due to these reasons, synthetic membranes used in OOC systems are also often coated with various ECM materials to better mimic the *in vivo* environment (Pasman et al., 2018). However, this also makes it more challenging to control the porosity and diffusion characteristics, thus communication between the two cell layers. In planar co-culture OOC designs, gel barriers and microchannel array act as porous membranes. To better mimic the *in vivo* basement membrane, these microchannels can be filled with ECM materials (Bryant-Greenwood, 1998; Richardson et al., 2017b). Often, optimization of the type and concentration of ECMs loaded into this microchannel array is required to ensure the proper control over molecular diffusion and cell migration.

For OOCs to mimic the feto-maternal interface, co-culture OOC designs are a perfect candidate to recapitulate its microarchitecture and functionality under physiological and pathological conditions since the feto-maternal interface is composed of seven different cellular layers, some with ECMs and some without ECMs (van Herendael et al., 1978; Bryant-Greenwood, 1998; Avila et al., 2014; Richardson et al., 2017b; Menon et al., 2018). Such co-culture OOC models can mimic the feto-maternal interface and enable a better understanding of their physiology as well as how they are affected by drugs, toxicants, or other biochemical signaling factors.

Fetal Membrane OOC (FM-OOC) Models

The first OOC to recreate components of the fetal membranes (i.e., the feto-maternal interface) was published in early 2019 (Richardson et al., 2019a). The FM-OOC was designed to better understand cellular interactions and paracrine cross-talk between maternal and fetal cells during pregnancy and parturition (Richardson et al., 2019a). The FM-OOC platform utilized the vertical co-culture OOC design and was composed of two orthogonal vertically stacked cell culture chambers containing equal surface areas. Primary AECs were seeded on top (fetal side), and primary decidual cells were placed on the bottom (maternal side). These chambers were separated by a semipermeable polycarbonate membrane (**Figure 5A**). The FM-OOC was utilized to detect membrane permeability, oxidative stress, and toxin-induced senescence, as well as cytokine production (**Figure 5A**). This device has many advantages over traditional transwell culture systems, including its ability to: (1) maintain physical and fluidic isolation between cell layers, (2) promote detectable biochemical changes, (3) better reproducibility, and (4) utilize fewer reagents and cells (Katt et al., 2016; Richardson et al., 2019a). However, it is still missing many vital components to recreate either the fetal membrane or the feto-maternal interface, such as (1) semipermeable membrane lacks ECM components to mimic that seen *in utero* (Richardson et al., 2019a), (2) organization of the device does not permit for imaging of both chambers, (3) direct imaging of migrating cells between chambers is not possible, where such cell migration is critical for understanding feto-maternal interface remodeling, and (4) lacks many cellular components of the feto-maternal interface, such as the AMCs, CMCs, and CTs (Richardson et al., 2017b, 2018b). Continuous advancement of FM-OOC models is expected, with the eventual goal to recreate the entire fetal membrane in OOC format. This device will be utilized to promote the study of cellular interactions during pregnancy and parturition, screening of drugs, and to advance research activities to reduce the risk of pregnancy-associated complications.

Amnion Membrane OOC (AM-OOC) Models

To address the imaging limitations of the previously developed FM-OOC model described above, as well as focus more on the amnion membrane, which alone contains two cellular layers (**Figure 2C**), recently an amnion membrane OOC (AM-OOC) system was developed, the first of its kind (Richardson et al., 2019b). The AM-OOC system utilizes a planar parallel co-culture OOC model design, having two circular culture chambers with interconnected microchannel array in between that functions as a controlled permeable barrier between the compartments (**Figure 5B**) (Richardson et al., 2019b). By culturing primary human AECs in the outer circular chamber and AMCs in the inner circular chamber, separated by Type IV collagen-filled microchannels mimicking the basement membrane, they were able to recreate the amnion membrane on an OOC format (Richardson et al., 2019b) (**Figure 5B**). Here, primary AECs and AMCs obtained from the midzones of term not in labor fetal membranes were utilized. This model was successfully utilized to show the interactive and transitional properties of amnion cells (epithelial-to-mesenchymal transition and mesenchymal-to-epithelial transition; Richardson and Menon, 2018; Richardson et al., 2018a, 2020b) under normal and oxidative stress conditions, similar to how they behave and respond *in utero* (Richardson et al., 2019b) (**Figure 5B**). Although this planar device allowed for easy cell imaging of both compartments as well as direct monitoring and tracking of migratory cells between compartments, there were still aspects that did not fully recreate the amnion membrane. For example, the AMCs were cultured in 2D, while *in utero* they are embedded in 3D collagen. Also, the system did not contain all of the four cell types and layers of the fetal membranes.

PERSPECTIVE

Proposed Ideas for a Full Feto-Maternal Interface OOC Model

Although the field currently has two established co-culture OOC models (Richardson et al., 2019a,b) (**Figure 5**), significant advances need to be made to better study and understand the feto-maternal interface as a whole using an OOC model. Some advanced fetal membrane models have recently been suggested, although not developed yet, to better recapitulate the fetal and maternal side of the feto-maternal interface (Gnecco et al., 2017). The proposed device contains four vertical chambers, each chamber containing AECs, trophoblast cells, decidua, and bacteria (Gnecco et al., 2017) (**Figure 6A**). Here, with the help of media perfusion, immune cells (i.e., macrophages and leukocytes) are envisioned to be added to the chorio-decidua layers of this model to recreate an

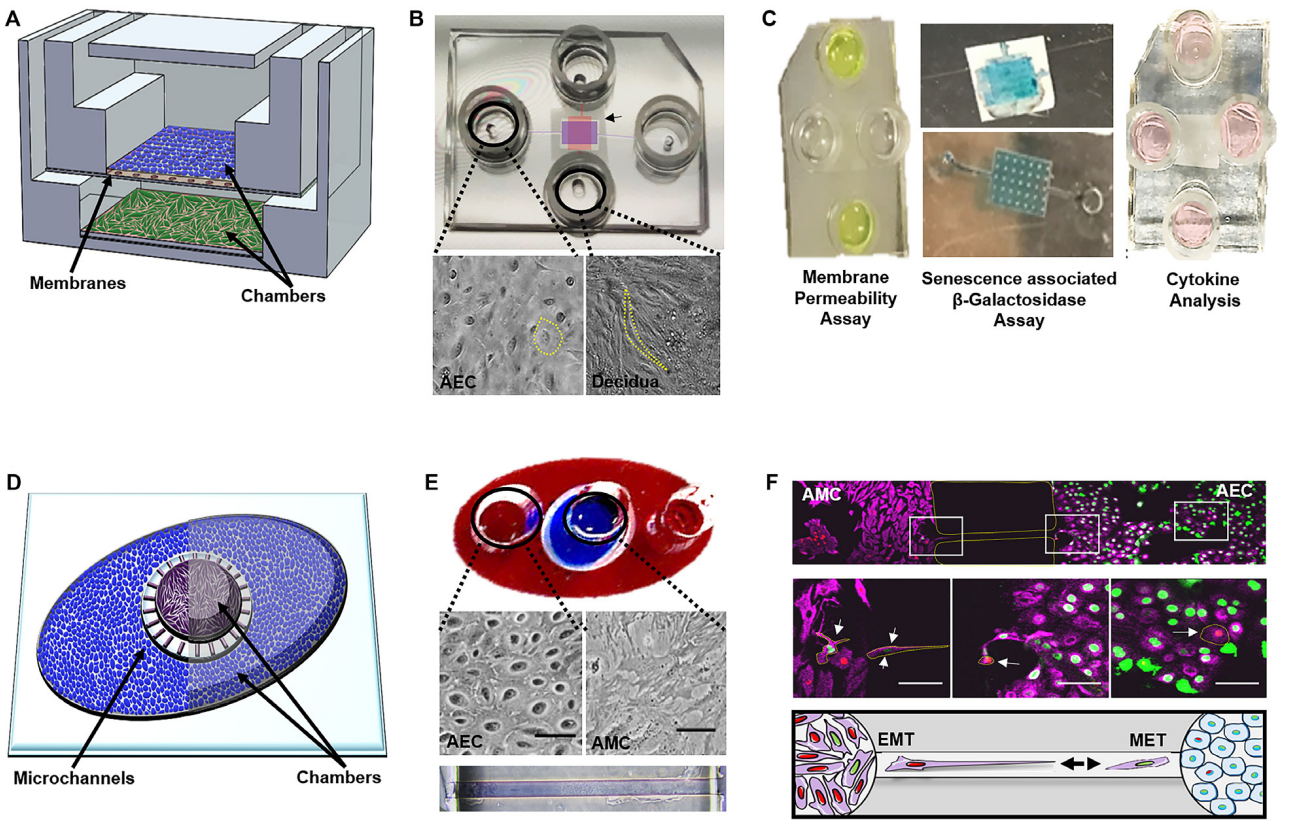

FIGURE 5 | Currently developed OOCs mimicking components of the fetal membranes and feto-maternal interface. **(A)** Device layout—schematic of the two-chamber fetal membrane-organ-on-chip (FM-OOC) developed to study fetal and maternal cell interactions at the fetal membrane interface adapted from Richardson et al. (2019a). The FM-OO-C is comprised of two stacked PDMS cell culture chambers that are coated with Matrigel (pink). Primary AECs (blue) are placed in the top chamber and grown on top of a polycarbonate semipermeable synthetic membrane, while primary decidual cells (green) are placed in the lower chamber and grown on the glass substrate. **(B)** Images of the fabricated FM-OO-C chips and cells being cultured within each compartment. Bright-field microscopy images of primary human AECs in the top chamber (purple) and primary decidual cells in the bottom chamber (red) are shown. The yellow outline visualizes the cellular morphology. **(C)** Endpoint assays—Left: Fluorescein isothiocyanate stain (yellow) is seen in the two horizontal columns feeding into the top AEC chamber. Media were collected from the bottom vertical columns to measure membrane permeability. Center: Bottom chamber showing representative senescence-associated β-galactosidase (SA-β-Gal) stained decidual cells and the semipermeable membrane containing blue staining representing SA-β-Gal + AECs. Right: Image of the FM-OO-C containing media from both amnion and decidual cells, which can be used to measure cytokine kinetics. **(D)** Device layout—the amnion membrane organ-on-chip (AM-OOC) is designed to recreate the amnion component of the fetal membrane by co-culturing AECs (blue) in an outer circular PDMS chamber and AMCs (purple) in the inner circular chamber. This planar two-chamber model is separated by a type IV collagen-filled (pink) microchannel array (mimicking the basement membrane). **(E)** The outer chamber of the AM-OOC was filled with red dye, and the inner chamber was filled with blue dye for visualization. Bright-field microscopy images of AEC morphology and AMC morphology inside an AM-OOC device. Microchannels filled with Type IV collagen Matrigel (stained with Masson trichrome), connecting the two culture chambers, are also shown. **(F)** Endpoint assay—confocal images showing native AECs (green) and AMCs (red), which have transitioned, migrated, and integrated into the opposite population. Middle right panel highlights (yellow) GFP-AECs that have migrated through the type IV collagen-filled microchannel, re-localized vimentin, and transitioned into a mesenchymal morphology indicative of EMT. Middle left panels highlight (yellow) RFP-AMCs that have migrated through the type IV collagen-filled microchannel, down-regulated vimentin, and transitioned into an epithelial morphology indicative of MET. The bottom panel is a schematic representing AECs (green) and AMCs (red) undergoing cellular transitions. Gray arrows highlight the migration direction. Pink, vimentin; green, histone 2B AEC; red, histone 2B AMCs. This figure is a rendition of Richardson et al. (2019b). All figures reused with permission.

infectious preterm birth model (Gnecco et al., 2017) (**Figure 6A**). Although this design takes into consideration the cell density ratio seen *in vivo*, it does not contain amnion or chorion mesenchymal cells within the ECM of the fetal membranes, nor does it recreate any ECM components or cell–collagen interactions (**Table 3**). However, it does, for the first time, propose a four-chambered OOC model and discuss the importance of immune cell activation in preterm birth; both of these components deem this OOC novel and ahead of its time in 2017.

An alternative FM-OOC model proposed here would utilize a four-chamber planner co-culture OOC design, culturing primary AECs, AMCs, CMSs/CTs, and decidua cells (**Figure 6B**). Interconnecting each culture chamber can be an array of microchannels that are filled with ECMs, recreating the amnion and chorion basement membrane. Additionally, AMCs and CMCs/CTs can be suspended in Matrigel and/or decellularized amnion collagen, creating 3D cultures in these compartments (**Figure 6B**). Such a model would utilize OOC technology to recreate the microarchitecture

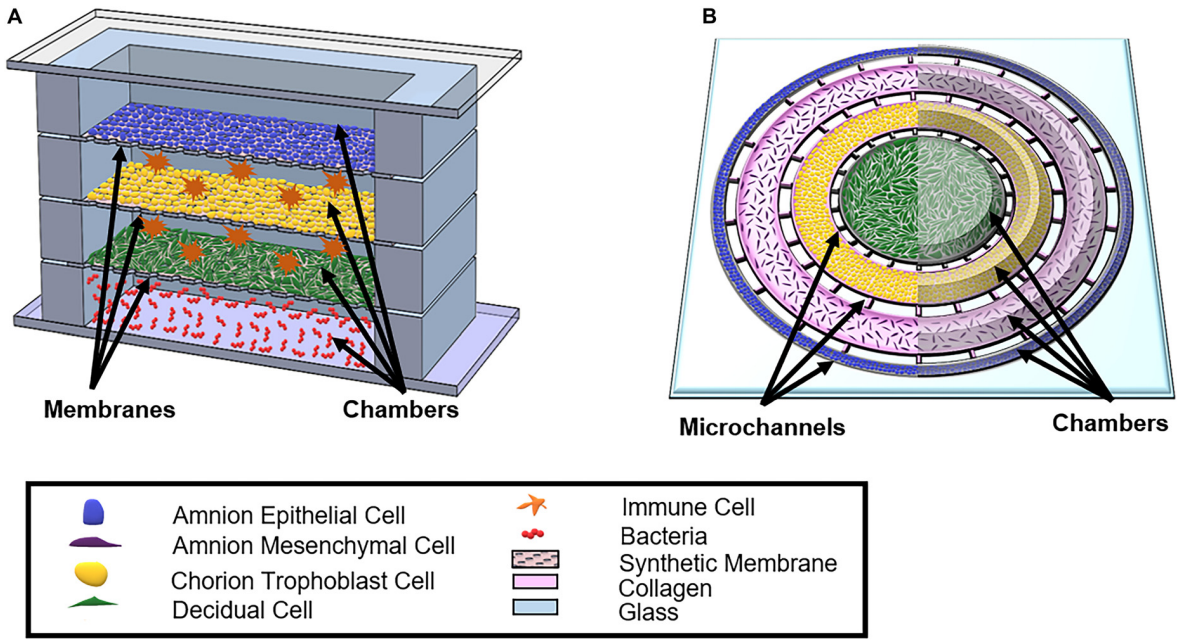

FIGURE 6 | Schematic of proposed OOCs better mimicking the full fetal membrane and feto-maternal interface. **(A)** A rendition of the proposed fetal membrane on a chip (IFMOC) by Gnecco et al. (2017) designed to create an infectious preterm birth model to study fetal membranes. This device contains four chambers culturing AECs (blue) on top, CTs (yellow) along with immune cells (orange) in the second chamber, decidua (green) and immune cells in the third chamber, and bacteria (red) in the bottom chamber. Each chamber is separated by a polycarbonate semipermeable synthetic membrane. **(B)** The proposed feto-maternal interface organ-on-chip (FMI-OOC) here is designed to mimic the feto-maternal interface, including the fetal membranes and maternal decidua. The FMI-OOC contains four co-centric circular cell culture chambers separated by arrays of microchannels. The cells are seeded following the *in vivo* structure; AECs (blue), AMCs (purple), CMCs/CTs (yellow), and decidua cells (green), respectively. Primary fetal membrane collagen and Matrigel (pink) can enable culturing AMCs and CMC/CTs in a 3D format. To recreate cell–collagen interfaces, microchannels can be filled with type IV collagen (pink) to mimic the basement membrane of the amnion and chorion layers, while the choriodecidua interface is left open (gray). All figures reused with permission. A comparison of both proposed OOC models can be found in **Table 3**.

of the feto-maternal interface down to every cell and collagen layer (Richardson et al., 2017b) (**Figure 2C** and **Table 3**). However, this device still lacks critical cellular components, including maternal and fetal immune cells, as well as the maternal layer of the decidua (parietalis). Integration of these cell layers along with biomechanical stressors (i.e., stretch) are needed in order to mimic the physiology of the feto-maternal interface.

Following the successful development of such a model, creating a pathologic condition of the feto-maternal interface, i.e., a disease OOC model, would be the next step. Such a disease model can mimic ascending and descending infection and inflammation, and be utilized to determine the propagation of infectious (e.g., lipopolysaccharides or bacteria) or inflammatory signals from maternal to fetal side, or vice versa, and test the efficacy of potential therapeutic compounds (i.e., anti-inflammatory molecules or synthetic drugs) in suppressing inflammation in each layer. Importantly, employing such a disease OOC model can contribute to the development of novel therapeutics against preterm birth, a very much needed area of developing considering that around 9.8% people in the United States alone are affected (Goldenberg et al., 2008; Liew et al., 2008; Beck et al., 2010; Blencowe et al., 2013; Lawn et al., 2013) while having the potential to significantly reduce the time and cost associated with pre-clinical and clinical trials. However, like any other model system, the developed OOCs also have several limitations, including: (1) each OOC is designed to answer certain biological questions, limiting their universal use, (2) requirements for specialized equipment to fabricate and conduct experiments, although this is not becoming easier, (3) have the tendency to be lower throughput, and (4) multi-organ chips are not available to model pregnancy.

Next Steps for Pregnancy-Related *in vitro* Methodologies

Although OOCs relating to the field of obstetrics are emerging over the past 5 years (Blundell et al., 2016, 2018; Lee et al., 2016; Gnecco et al., 2017; Nagashima et al., 2018; Pemathilaka et al., 2019a,b; Richardson et al., 2019a,b; Yin et al., 2019), significant future research is needed in order to truly create an *in vitro* pregnancy model to better understand feto-maternal communication, the induction of term and preterm labor, and drug or toxicant permeability at these vital interfaces. Advances from traditional 2D culture systems to novel 3D culture platforms are contributing to overcoming these knowledge gaps. 3D cell culture, typically referred to as an organoid culture [i.e., cell spheroids (Okere et al., 2015), cell sheets (Richardson et al., 2020a), or tissue printing (Kang et al., 2016)], utilizes cell

TABLE 3 | Characteristics of the proposed OOCs compared to *in vitro* and *in vivo* conditions.

Characteristics	Cell type	2D cell culture	3D cell culture	IFMOC	FMI-OOC	Mice models	Non-human primate	Human
Morphology	AEC	Cuboidal/fibroblastoid	Fibroblastoid,	Cuboidal/fibroblastoid	Cuboidal/fibroblastoid	Cuboidal/fibroblastoid	Cuboidal/fibroblastoid	Cuboidal/fibroblastoid
	AMC	Fibroblastoid	Cuboidal/fibroblastoid,	Not present,	Fibroblastoid	Fibroblastoid	Fibroblastoid	Fibroblastoid
	CMC/CT	Fibroblastoid/cuboidal	Fibroblastoid/cuboidal	Only contain cuboidal CTs,	Fibroblastoid/cuboidal	Only contain cuboidal CTs,	Fibroblastoid/cuboidal	Fibroblastoid/cuboidal
	DECI	Fibroblastoid	Fibroblastoid	Fibroblastoid	Fibroblastoid	Not connected,	Fibroblastoid	Fibroblastoid
Collagen production	AEC	Low,	High	High	High	High	High	High
	AMC	High	High	High	High	High	High	High
	CMC/CT	Low,	High	High	High	High	High	High
	DECI	Low	Low	Low	Low	Low	Low	Low
Intermediate filament expression	AEC	Metastate	Mesenchymal	Metastate	Metastate	Metastate	Metastate	Metastate
	AMC	Mesenchymal	Metastate	Not present,	Mesenchymal	Mesenchymal	Mesenchymal	Mesenchymal
	CMC/CT	Mesenchymal/epithelial	Mesenchymal/epithelial	Only contain epithelial CTs,	Mesenchymal/epithelial	Only contain epithelial CTs,	Mesenchymal/epithelial	Mesenchymal/epithelial
	DECI	Mesenchymal	Mesenchymal	Mesenchymal	Mesenchymal	Not connected,	Mesenchymal	Mesenchymal

Red font indicates differences.

aggregates either with single cell type or multiple cell types, often together with various ECMs, to recreate components of the fetal membrane and feto-maternal interface (Liu and Qi, 2010; Davydova et al., 2011; Shieh et al., 2017). While 3D growth of cells has been documented in many fields to induce expression of more *in vivo* characteristics and functionality, only a handful of studies have been conducted with fetal membrane-derived cells. Importantly, no attempts have been made so far to recreate the fetal membrane or feto-maternal interfaces using such 3D bioprinting techniques that have been utilized to print volumetric shapes of cell and collagen layers to recreate ear, noses, and eye components (Kuru et al., 2016; Isaacson et al., 2018; Jodat et al., 2020). The advantages and limitations of each of these 3D culture techniques are also summarized in **Table 1**.

Impact to Clinical Research

Although many clinical studies have been conducted evaluating different aspects of fetal membranes, it is still challenging under certain settings to acquire approval and or recruit enough patients within individual clinical conditions (i.e., preeclampsia, pPROM, chorioamnionitis, gestational diabetes) in order to provide tissue for basic research and/or to conduct clinical trials [162, 163]. A "pregnancy-on-chip" platform that can represent various pathologic conditions of pregnancy could provide a useful model to conduct clinical trials that generally could not occur. This model is also ideal for testing FDA-approved drugs that currently do not contain enough pre-clinical data related to transport across the feto-maternal interfaces. This cost-effective approach could lead to the approval of dozens of drugs to be repurposed toward treating pregnancy-related complications. Additionally, OOC-based studies can be adapted to clinical research to conduct experiments that can lead to better understand the mechanism of drug functions (e.g., efficacy, cytotoxicity, passage through distinct layers of the feto-maternal interfaces), and be utilized for pre-clinical trials of therapeutic development against preterm birth, or even replace part of a clinical trial.

Besides, the recent European Union (EU) ban on animal testing for cosmetic products (No Author, 2013), as well as US EPA's current directive that prioritizes efforts to reduce animal testing, are expected to further spur this area. The development of novel OOC models suggests two new promising concepts: (1) "personalized medicine-on-chip" by using patient-derived cells, including primary cells and inducing patient-derived fat cells into iPSCs, which can consider the effect of patient-to-patient variability (Jodat et al., 2018; van den Berg et al., 2019) and (2) applying the "human-on-chip" concepts to clinical trials (Luni et al., 2014; Abaci and Shuler, 2015; Maschmeyer et al., 2015).

Impact on Basic Research

The many challenges in this area are not just faced clinically but also while conducting basic biological research. Current, *in vitro* (i.e., 2D, 3D, or transwell culture) and *ex vivo* (i.e., explant culture) assays provide complexity in understanding multi-organ communication between individual cellular or collagen layers within the tissue and or organ-to-organ systems (**Tables 1**, **3**). Understanding this communication is vital to answering

physiological questions related to gestation, term, and preterm parturition, as well as the pharmaceutical questions related to pre-clinical trials. Furthermore, understanding individual cellular contribution to labor onset and adverse pregnancy outcomes could potentially identify novel biomarkers of term and preterm delivery. Biomarkers identified in this manner could lead to the development of standard clinic testing for patients having a risk for preterm labor.

CONCLUSION

Organ-on-chips represent a variety of physiological and pathophysiological states of diverse organ structures that are contributing to a better understanding of complex organ systems. These platforms also have the potential to become critical steps in the drug discovery pipeline, as well as part of the future of bench to bedside research. Although the importance of the feto-maternal interface in pregnancy has been documented for decades, only recently has the technology allowed for novel *in vitro* techniques to recreate the anatomy and function of the placenta and fetal membranes accurately. Several placenta-on-chip (Blundell et al., 2016, 2018; Lee et al., 2016; Pemathilaka et al., 2019a,b; Yin et al., 2019) and fetal membrane-on-chip (Richardson et al., 2019a,b) platforms have emerged by mimicking the microarchitecture and functions of both feto-maternal interfaces, and are improving our understanding of this vital organ system. As more advanced OOC models of the feto-maternal interface emerge, we expect such models to radically change how research and development are conducted in the field of obstetrics.

AUTHOR CONTRIBUTIONS

LR and SK drafted the manuscript and created the figures, while AH and RM reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Proteomic Study of Fetal Membrane: Inflammation-Triggered Proteolysis of Extracellular Matrix May Present a Pathogenic Pathway for Spontaneous Preterm Birth

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Introduction: Spontaneous preterm birth (sPTB), which predominantly presents as spontaneous preterm labor (sPTL) or prelabor premature rupture of membranes (PPROM), is a syndrome that accounts for 5–10% of live births annually. The long-term morbidity in surviving preterm infants is significantly higher than that in full-term neonates. The causes of sPTB are complex and not fully understood. Human placenta, the maternal and fetal interface, is an environmental core of fetal intrauterine life, mediates fetal oxygen exchange, nutrient uptake, and waste elimination and functions as an immune-defense organ. In this study, the molecular signature of preterm birth placenta was assessed and compared to full-term placenta by proteomic profiling.

Materials and Methods: Four groups of fetal membranes (the amniochorionic membranes), with five cases in each group in the discovery study and 30 cases in each group for validation, were included: groups A: sPTL; B: PPROM; C: full-term birth (FTB); and D: full-term premature rupture of membrane (PROM). Fetal membranes were dissected and used for proteome quantification study. Maxquant and Perseus were used for protein quantitation and statistical analysis. Both fetal membranes and placental villi samples were used to validate proteomic discovery.

Results: Proteomics analysis of fetal membranes identified 2,800 proteins across four groups. Sixty-two proteins show statistical differences between the preterm and full-term groups. Among these differentially expressed proteins are (1) proteins involved in inflammation (HPGD), T cell activation (PTPRC), macrophage activation (CAPG, CD14, and CD163), (2) cell adhesion (ICAM and ITGAM), (3) proteolysis (CTSG, ELANE, and MMP9), (4) antioxidant (MPO), (5) extracellular matrix (ECM) proteins (APMAP, COL4A1, LAMA2, LMNB1, LMNB2, FBLN2, and CSRP1) and (6) metabolism of glycolysis (PKM and ADPGK), fatty acid synthesis (ACOX1 and ACSL3), and energy biosynthesis (ATP6AP1 and CYBB).

Conclusion: Our molecular signature study of preterm fetal membranes revealed inflammation as a major event, which is inconsistent with previous findings. Proteolysis may play an important role in fetal membrane rupture. Extracellular matrix s have been altered in preterm fetal membranes due to proteolysis. Metabolism was also altered in preterm fetal membranes. The molecular changes in the fetal membranes provided a significant molecular signature for PPROM in preterm syndrome.

Keywords: fetal membrane, preterm birth, prelabor premature rupture of membrane, inflammation, extracellular matrix

INTRODUCTION

Preterm birth refers to infants born alive before 37 weeks of gestation (Quinn et al., 2016). Preterm birth accounts for 5–12% of all live births worldwide (Gezer et al., 2018). As a populated country, China has about 1.17 million preterm infants birth every year (World Health Organization [WHO], 2018). In particular, since 2016, when the Chinese government relaxed the one-child policy and implemented its two-children policy, many women of high maternal age rushed to have a second child (Cheng and Duan, 2016). It has been demonstrated that pregnancy at 40 years of age and older is strongly associated with preterm birth and other disorders of pregnancy (Fuchs et al., 2018). In preterm newborns, the brain, lung, liver, and other organs are not fully developed, and there is a high incidence of brain injury, neonatal respiratory distress syndrome, bilirubin encephalopathy, and multiple organ failure (Fraser et al., 2004; Bhutani and Wong, 2013; Paton et al., 2017). It is estimated by the WHO that preterm birth is the most common cause of children's death under the age of 5 years (World Health Organization [WHO], 2018). Preterm birth brings a great economic burden to families, communities, and societies.

The placenta serves as the interface between the pregnant mother and the intrauterine fetus, with the main function of exchanging the material between the fetus and the mother, providing the oxygen and nutrients required for embryonic, as well as fetal, development, and excreting metabolic waster and CO_2 (Gude et al., 2004). In addition, the placenta also serves as a barrier to bacteria, pathogens, and drugs because of the existence of the placental barrier (Zeldovich et al., 2013). The placenta can also synthesize chorionic gonadotropin, human placental lactogen (hPL), estrogen, progesterone, cytokine, and growth factors (Costa, 2016). In addition, the placenta has immune tolerance to the fetus (Guleria and Sayegh, 2007). Therefore, abnormal placental function has a direct correlation with the occurrence of preterm birth. So far, estrogens, hPL, placenta growth factor (PLGF), human chorionic gonadotrophin (hCG), plasma protein A (PAPP-A), placental protein 13 (PP-13), pregnancy-specific glycoproteins, and progesterone metabolites have been employed as surrogate markers of placental function (Heazell et al., 2015). Despite recent progress on the study of these markers associated with function of placenta, a comprehensive and systemic understanding of the pathophysiology of the placenta is lacking, particularly little is known about the pathogenic role of fetal membrane and its involvement in the development of spontaneous preterm birth (sPTB). Here, we studied the protein expression of fetal membrane, with the aim to understand the pathophysiology of fetal membrane and to identify novel molecules associated with preterm birth in the fetal membrane. This study not only provides theoretical support for the occurrence of preterm birth, but also provides reference for the early diagnosis and early intervention of premature birth.

MATERIALS AND METHODS

Specimens

The study was approved by the Hospital Ethics Committee of Sanya Maternity and Child Care Hospital and the informed consent was obtained from pregnant women to permit the use of placentas in research studies. A retrospective study was designed to investigate the pathological alteration of protein expression with a pre-banked birth cohort of 20 placentas, which were grouped as (A): spontaneous preterm labor (sPTL), defined as a non-medical and/or non-selective spontaneous birth delivered between 20^{+1} and 36^{+6} gestational week (GW) in which regular contractions of the uterus result in changes in the cervix before 37 weeks of pregnancy, (B): prelabor premature rupture of membranes (PPROM) that occurred between 20^{+1} and 36^{+6} GW, (C): full-term birth (FTB) between 39^{+1} and 40^{+6} GW, and (D): full-term premature rupture of membrane (PROM). There were five placentas in each group. The placentas were collected from fetal membranes through full layers to the decidua. Fetal membranes were dissected within 2 cm of the edge where the membrane naturally ruptured during labor. However, if a rupture hole could be identified in the premature rupture (PPROM and PROM) cases, the membrane would be collected within 1 cm around the hole where the premature rupture occurred.

Criteria for Inclusion and Exclusion

Tissues selected from prebanked samples had to meet the following criteria: (i) age of the pregnant woman is 18–45 years, (ii) no clinically recognized infection/inflammation (INF) before and/or during pregnancy (INF is determined by phenotypically notable fever, increased counts of peripheral white blood cells, and/or increased IL6 and/or TNFα), (iii) primipara and singleton without history of miscarriage or abortion, (iv) vaginal delivery with (PPROM and PROM) or without (FTB and sPTL) premature rupture of chorioamniotic membrane,

TABLE 1 | Demographic and clinical information about placentas.

Group	A	B	C	D
Birth	sPTL	PPROM	FTB	PROM
Pregnant age (year old)	22-28	22-30	22-25	22-26
Gestational age (weeks)	29^{+3}–31^{+6}	30^{+0}–32^{+1}	40^{+0}–40^{+6}	39^{+0}–40^{+6}
Primipara	Yes	Yes	Yes	Yes
Singleton	Yes	Yes	Yes	Yes
Mode of delivery	Spontaneous	Spontaneous	Spontaneous	Spontaneous
Family history of preterm birth	No	No	No	No
Family history of birth defect	No	No	No	No
Infection history during pregnancy	No	No	No	No
Gestational complication	No	No	No	No
Use of antibiotics during pregnancy	No	No	No	No
Use of steroid	No	No	No	No
Inform consent obtained	Yes	Yes	Yes	Yes

(v) no vaginal bleeding during the pregnancy, (vi) no other pregnancy-related complication(s) and no clinical intervention with antibiotics, steroids, or tocolytics during the pregnancy, (vii) no family history of birth defects, and (viii) no consanguinity. Any cases not meeting the above criteria were excluded. Details of demographic and clinical information for the samples studied are provided in **Table 1**.

Protein Extraction From Placenta Membrane for Proteomic Analysis

The human placental amniochorionic membrane, or fetal membrane, was dissected (20- to 30-mg cross-sections) and first washed with cold PBS containing protease inhibitor (Sigma-Aldrich, United Kingdom), then placed in beads-beater tubes containing RIPA lysis buffer (Thermo Fisher Scientific, United Kingdom) to make it 50 mg/ml. Tissues were homogenized four times at 6,500 Hz for 40 s in a beads-beater (Stretton, United Kingdom) (**Figure 1**). The samples were centrifuged at 10,000 g for 5 min at 4°C to remove insoluble tissue debris. The protein concentration in the homogenates was determined by BCA assay (Thermo Fisher Scientific, United Kingdom), and 100 μg of total proteins were added to a 30-kDa filter (Merck Millipore, United Kingdom). Proteins were reduced by 10 mM Dithiothreitol (DTT) (Sigma, United Kingdom) at 37°C for 1 h and then alkylated with 40 mM iodoacetamide (IAA, Sigma, United Kingdom) for 45 min in the dark, at room temperature. Samples were centrifuged for 20 min at 14,000 g to remove DTT and IAA, followed by buffer exchange with 8 M urea twice and 50 mM ammonia bicarbonate three times. One hundred microliters of trypsin were added at a trypsin/protein ratio of 1:50 for digestion at 37°C overnight. Digested peptides were collected by upside down spin, and membrane filters were washed twice with 0.5 M NaCl and water, respectively. The peptides were purified by a SepPak C18 cartridge (Waters, United Kingdom), dried by SpeedVac centrifugation, and resuspended in buffer A (2% acetonitrile, 0.1% formic acid) for LC-MS/MS analysis.

Peptide Measurement by Mass Spectrometry

LC-MS/MS analysis was carried out by nano-ultra performance liquid chromatography tandem mass spectrometry analysis using a 75-μm-inner diameter × 25 cm C18 nanoAcquity UPLC column (1.7-μm particle size, Waters, United Kingdom). Peptides were separated with a 120-min gradient of 3–40% solvent B (solvent A: 99.9% H_2O, 0.1% formic acid; solvent B: 99.9% ACN, 0.1% formic acid) at 250 nl/min and injected into a Q Exactive High Field (HF) Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, United Kingdom) acquiring data in electron spray ionization (ESI) positive mode. The MS survey was set with a resolution of 60,000 FWHM, with a recording window between 300 and 2,000 m/z. A maximum of 20 MS/MS scans were triggered in data-dependent acquisition (DDA) mode.

Protein Identification and Quantification

MaxQuant software (v1.5.8.3, Max Planck Institute of Biochemistry, Germany) was used for peptide and protein identification and quantitation. Data generated from MS/MS spectra were searched against the Uniprot human database (version 2017); 20,205 entries were used for peptide homology identification. The false discovery rate (FDR) was set to 1% for protein and peptide identification. Proteins were quantified by at least one unique peptide, and match between run was selected to increase the quantifiable value cross samples (**Figure 1**). Label-free quantitation (LFQ) intensity data were used for further analysis and comparisons across the variant groups. Statistical analysis was assessed by using Persus software (version 1.5.5.3, Max Planck Institute of Biochemistry). Statistical comparisons between the groups were performed by using two-sided unpaired Student's t-test. Firstly, threshold p-value was used to define statistical significance. Secondly, permutation-based FDR was used to assess truncate data.

Validation of Differentially Expressed Proteins With Western Blot

Ten milligrams of fetal membranes or placental villi were dissected and lysed in RIPA buffer containing protease inhibitors (Roche, United States). Western blot analysis was performed by loading 15 μg of proteins on 4–12% pre-cast Bis-Tris gels (Bio-Rad, United States) and transferred to PVDF membranes (Merck Millipore, United States). Membranes were incubated with mouse anti-human MPO monoclonal antibody (Santa Cruz, United States, 1:200 diluted in 2% milk), mouse anti-human elastase, neutrophil expressed (ELANE) monoclonal antibody

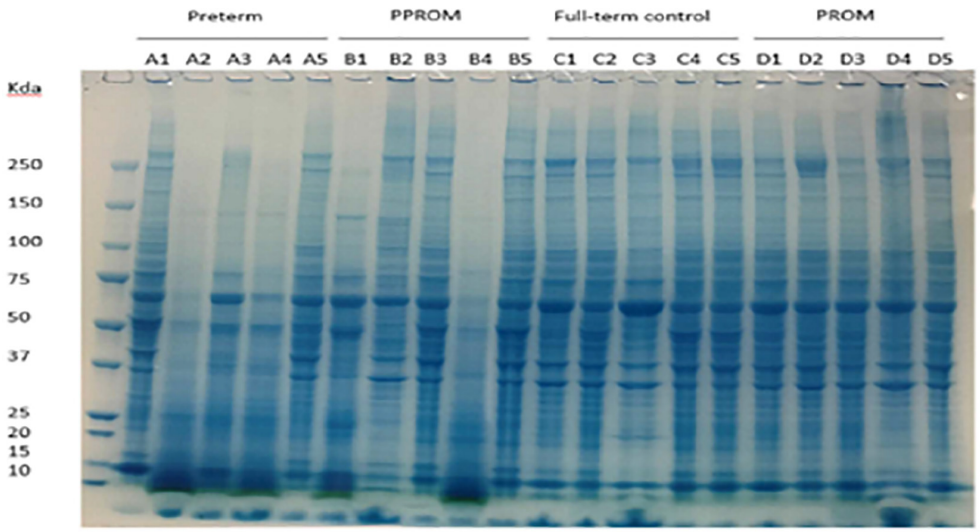

FIGURE 1 | Protein expression pattern on SDS-PAGE gel from four groups of fetal membrane. Group A: sPTL (preterm), group B: PPROM, group C: FTB, and group D: PROM. Five individual samples in each group were analyzed on an 8% SDS-PAGE stained with a dye of Instantblue.

(Santa Cruz, United States, 1:200 diluted in 2% milk), mouse anti-human GAPDH monoclonal antibody (Thermo Fisher Scientific, United Kingdom, 1:2,000 diluted in 2% milk), rabbit anti-human beta-actin antibody (Abcam, United Kingdom, 1:1,000 diluted in 2% milk). Dye-800-conjugated secondary antibodies were applied and visualized with an Odyssey Clx (Li-Cor, United States). Image studio (Li-Cor, United States) and Image J (Schneider et al., 2012) were used for Western blot quantitation, and one-way ANOVA was used for statistical significance test.

RESULTS

Quality Control of Proteins Isolated From the Fetal Membrane Tissues

Protein lysates of fetal membranes were run on SDS-PAGE and visualized with InstantBlue (**Figure 1**). Protein partial degradation was detected in samples of A2, A4 and B1, B4. The protein patterns of the rest of samples did not show a clear difference.

Differentially Expressed Proteins Identified From Mass Spectra

After LC-MS/MS measurement, Maxquant analysis identified a total of 2,880 proteins from fetal membrane samples. The hierarchical clustering analysis indicated protein abundance alteration between individual samples. In addition to comparisons of the preterm group (A + B) with the full-term control group (C + D), individual comparisons were applied to identified specific protein(s) that are associated with a specific condition (**Figure 2**). Protein differential expression was defined by the criteria of $p < 0.01$, fold change (FC) ≥ 2, and permutation-based FDR 0.05, with which, 62 (38 up-regulated and 24 down-regulated) proteins were identified to be differentially expressed (**Tables 2a**,**b**). Among these 62 proteins, 20 were identified to be the top-listed FC (four were down-regulated and 16 were up-regulated). The FCs of 8 of 20 up-regulated proteins were >11, among which MMP9 showed 318.64 FC as the highest (**Table 3**). All differentially expressed proteins identified from fetal membranes in sPTB (sPTL and PPROM) were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis to identify the pathways that may be associated with the sPTB. The top-scoring pathways were infection and inflammation, protein degradation and proteolysis, extracellular matrix (ECM), cell adhesion, antioxidant, glycolysis, and fatty acid (FA) oxidation (**Table 4**).

Validation of Differentially Expressed Proteins With Western Blots

Three proteins, MPO, ELANE, and GAPDH, which were shown by LC-MS/MS to be differentially expressed in fetal membrane, were randomly selected to validate the differential expression value of protein with Western blots. Westerns blot of MPO and ELANE showed the same protein expression pattern as proteome data with statistical significance. While GAPDH followed a similar increase of protein expression in fetal membrane compared to placental villi, it had a decreased expression in fetal membrane of preterm cases, including both sPTL and PPROM, compared to FTB and PROM (groups C and D) (**Figure 3**).

DISCUSSION

Placental Function and Preterm Birth

PTB plays a significant adverse impact on the increased mortality and mobility of preterm-born neonates. The etiology

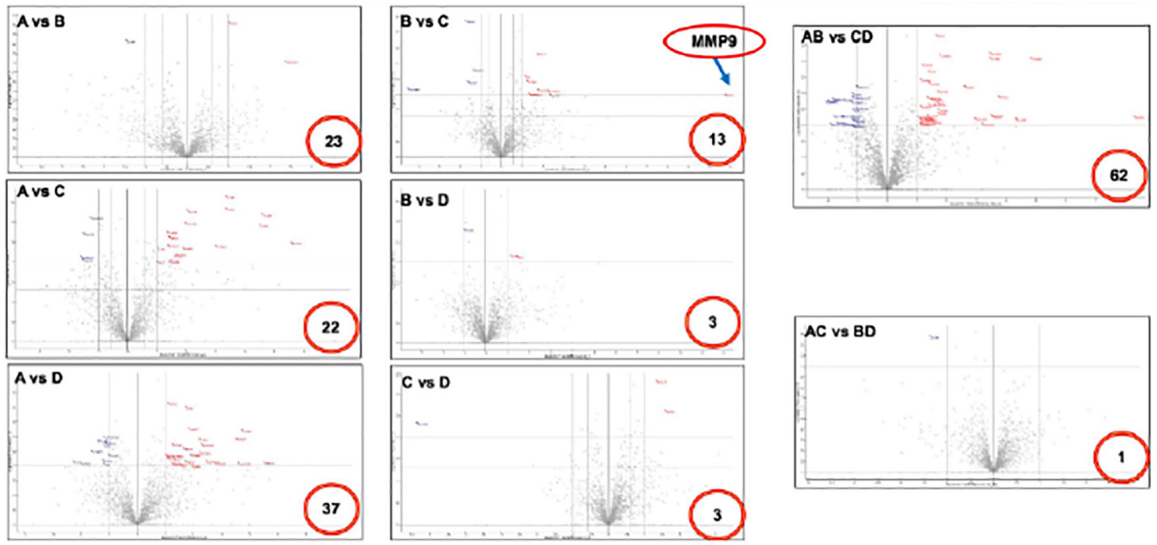

FIGURE 2 | Volcano plot of differentially expressed proteins in fetal membranes. Various comparisons identified specific protein(s) that associated with the premature condition. Number in the red circle indicates the number of differential proteins with statistical significance. *X*-axis indicates fold changes and *Y*-axis indicates *p* value-log Student *t*-test. A: sPTL, B: PPROM, C: FTB, and D: PROM.

of sPTB is multi-factorial; however, placental dysfunction has been identified as the leading cause of premature birth due to it pivotal role between the fetus and mother during pregnancy (Audette and Kingdom, 2018). As a result of the pathophysiological changes in placental dysfunction, including poor trophoblast uterine invasion and impaired transformation of the uterine spiral arteries to high capacity and low impedance vessels, which eventually leads to lower blood flow to the placenta (Ilekis et al., 2016; Cuffe et al., 2017), the placenta was unable to sustain fetal development requirements and thus preterm birth occurred. Searches for molecular markers to predict preterm birth have been conducted mainly in the maternal blood due to its richness of information and easy accessibility. It has been reported that PP-13 and PAPP-A are good predictors of preterm birth (Stout et al., 2013). Elevated maternal serum–soluble fms-like tryrosine 1 (sFlt1), inflammation marker [cysteine-rich protein (CRP)], and PIGF are associated with preterm birth (Bastek et al., 2011; Straughen et al., 2012). However, these markers have little clinical use for the prediction of preterm birth or for understanding the pathophysiology of placental dysfunction. Therefore, we conducted an unbiased proteomic analysis of the placenta in preterm birth compared to in full-term controls, followed by various comparisons to generate differentially expressed proteins specific to one single variation. Such comparisons include group B vs. group C, for example, in which the outcome of differentially expressed proteins was influenced only by premature rupture of fetal membrane in PPROM (B) vs. FTB without rupture of membrane (C). In addition, such comparisons may reduce non-specific noise, such as the comparison of group A vs. group D, in which the differentially expressed proteins would likely have resulted from the premature labor that was related to muscle contraction of uterine but not to rupture of membrane.

Proteins Involved in Infection and Inflammation

Based on molecular function, the top-scoring proteins differentially expressed in fetal membranes of sPTB have been clustered into five groups *via* their involvement in pathophysiological pathways (**Table 3**). The main pathophysiological changes in the fetal membrane from preterm birth were inflammation. Numerous studies indicated that placental intrauterine infection was strongly associated with sPTL or PPROM (Salafia et al., 1991; Helmig et al., 2002; Morgan, 2016; Pugni et al., 2016; Chisholm et al., 2018).

A study in humans demonstrated that HPGD expression in preterm-labor placenta tissues was decreased in comparison with a full-term labor group (He et al., 2015). HPGD is a member of the short-chain non-metalloenzyme alcohol dehydrogenases protein family and is responsible for the degradation of prostaglandins, hormones that modulate the inflammatory response (Aoki and Narumiya, 2012; Seo and Oh, 2017). Another study in an animal model detected high expression levels of HPGD at the beginning and at normal term of pregnancy, indicating that HPGD may play a role during the establishment and termination of gestation (von Hof et al., 2017). Our data are consistent with previously findings. The lower level of HPGD found in fetal membranes suggested a higher level of inflammatory states in the preterm group (sPTL + PPROM), possibly through negative regulation of inflammatory molecule prostaglandins.

PTPRC is a receptor-type protein tyrosine phosphatase that regulates cell growth, differentiation, and mitosis. PTPRC is

TABLE 2a | Proteins identified from fetal membranes associated with preterm birth (AB vs. CD): up-regulated.

Majority protein IDs	Protein names	Gene names	Protein family	Pathways	Functions
Q9HDC9; H0Y512; Q9HDC9-2	Adipocyte plasma membrane-associated protein	APMAP	Adipocyte plasma membrane-associated protein (PTHR10426:SF26)		
Q15904; A0A0C4DGX8	V-type proton ATPase subunit S1	ATP6AP1	V-type proton ATPase subunit S1 (PTHR12471:SF2)	Energy metabolism	ATP synthase
Q93050-1; Q93050; Q93050-3; B7Z641; B7Z2A9; F5H1T6	V-type proton ATPase 116 kDa subunit a isoform 1; V-type proton ATPase subunit a	ATP6V0A1	V-type proton ATPase 116 kDa subunit a isoform 1 (PTHR11629:SF68)	Energy metabolism	ATP synthase
F5GYQ1; P61421; J3QL14; R4GN72	V-type proton ATPase subunit d 1	ATP6V0D1	V-type proton ATPase subunit d 1 (PTHR11028:SF3)	Energy metabolism	ATP synthase
A6NC48; Q10588; H0Y984; Q10588-2	ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2	BST1	ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2 (PTHR10912:SF4)		
A0A0A0MSV6; D6R934; P02746; D6RGJ1	Complement C1q subcomponent subunit B	C1QB	Complement C1q subcomponent subunit B (PTHR44403:SF2)		NAD metabolism and Innate Immune System
B4DPQ0; P00736; F5H2D0	Complement C1r subcomponent; Complement C1r subcomponent heavy chain; Complement C1r subcomponent light chain	C1R	Complement C1r subcomponent (PTHR45206:SF1)		
P08571; D6RFL4	Monocyte differentiation antigen CD14; Monocyte differentiation antigen CD14, urinary form; Monocyte differentiation antigen CD14, membrane-bound form	CD14	Monocyte differentiation antigen CD14 (PTHR10630:SF3)	Toll receptor signaling pathway (P00054)	
F5GZZ9; Q86VB7-3; Q86VB7; Q86VB7-2; C9JHR8; Q86VB7-4	Scavenger receptor cysteine-rich type 1 protein M130; Soluble CD163	CD163	Scavenger receptor cysteine-rich type 1 protein M130 (PTHR19331:SF392)	Macrophages function	Acute phase-regulated receptor involved in clearance and endocytosis of hemoglobin/haptoglobin complexes by macrophages
H0YD13; P16070-18; P16070-12; P16070-14; P16070-13; P16070-11; P16070-10; P16070-16; P16070-8; P16070-17; P16070-6; P16070-4; P16070-3; P16070-7; P16070-5; P16070; H0Y2P0; H0YE40	CD44 antigen	CD44	CD44 antigen (PTHR10225:SF6)		
F8VNT9; F8VV56; F8W022; F8VWK8; P08962-3; P08962-2; P08962	Tetraspanin; CD63 antigen	CD63	CD63 antigen (PTHR19282:SF233)		
P02452	Collagen alpha-1 (I) chain	COL1A1	Collagen alpha-1(I) chain (PTHR24023:SF569)	Integrin signaling pathway (P00034)	Extracellular matrix structural constituent
P02462-2; P02462	Collagen alpha-1(IV) chain; Arresten	COL4A1	Collagen alpha-1(IV) chain (PTHR24023:SF854)	Integrin signaling pathway (P00034)	Extracellular matrix structural constituent

(Continued)

TABLE 2a | Continued

Majority protein IDs	Protein names	Gene names	Protein family	Pathways	Functions
P08311	Cathepsin G	CTSG	Cathepsin G (PTHR24271:SF13)	Protein degradation	Proteolysis
P04839	Cytochrome b-245 heavy chain	CYBB	Cytochrome b-245 heavy chain (PTHR11972:SF60)	Energy metabolism	ATP synthase/oxidase
P08246	Neutrophil elastase	ELANE	Neutrophil elastase (PTHR24257:SF16)	Protein degradation	Proteolysis/phagocytosis
A0A0D9SEN1;Q12884; B4DLR2	Prolyl endopeptidase FAP; Antiplasmin-cleaving enzyme FAP, soluble form	FAP	Prolyl endopeptidase FAP (PTHR11731:SF136)	UPS	Ubiquitin-protein ligase activity (ubiquitin proteasome system)
P98095-2; P98095	Fibulin-2	FBLN2	Fibulin-2 (PTHR44887:SF1)		
Q86UX7-2; Q86UX7;F5H1C6	Fermitin family homolog 3	FERMT3	Fermitin family homolog 3 (PTHR16160:SF1)		
P02792	Ferritin light chain	FTL	Ferritin light chain (PTHR11431:SF47)		
P11413; P11413-3; P11413-2; E9PD92; E7EM57; E7EUI8	Glucose-6-phosphate 1-dehydrogenase	G6PD	Glucose-6-phosphate 1-dehydrogenase (PTHR23429:SF0)	Glycolysis	Glycolysis
P05204; A0A087WZE9; Q15651-2; Q15651	Non-histone chromosomal protein HMG-17; High mobility group nucleosome-binding domain-containing protein 3	HMGN2;HMGN3			
P05362; K7EKL8	Intercellular adhesion molecule 1	ICAM1	Intercellular adhesion molecule 1 (PTHR13771:SF9)		Cell adhesion signaling
P11215; P11215-2	Integrin alpha-M	ITGAM	Integrin alpha-M (PTHR23220:SF120)	Inflammation mediated by chemokine and cytokine signaling pathway	
(P00031)/Integrin signaling pathway (P00034)					
A0A087WX80; P24043; A0A087WYF1	Laminin subunit alpha-2	LAMA2	Laminin subunit alpha-2 (PTHR10574:SF291)	Integrin signaling pathway (P00034)	Extracellular matrix linker protein receptor
P20700; E9PBF6; A0A0D9SFE5	Lamin-B1	LMNB1	Lamin-B1 (PTHR23239:SF157)	FAS signaling pathway (P00020)	Structual molecule activity
Q03252	Lamin-B2	LMNB2	Lamin-B2 (PTHR23239:SF152)	FAS signaling pathway (P00020)	Structual molecule activity
P14780	Matrix metalloproteinase-9; 67 kDa matrix metalloproteinase-9; 82 kDa matrix metalloproteinase-9	MMP9	Matrix metalloproteinase-9 (PTHR10201:SF30)	Plasminogen activating cascade (P00050)/CCKR signaling(P06959)	Collagenases (degrade collagen)
P05164-2; P05164; P05164-3	Myeloperoxidase; Myeloperoxidase; 89 kDa myeloperoxidase; 84 kDa myeloperoxidase; Myeloperoxidase light chain; Myeloperoxidase heavy chain	MPO	Myeloperoxidase (PTHR11475:SF108)		Antioxidant (GO:0016209)

(Continued)

TABLE 2a | Continued

Majority protein IDs	Protein names	Gene names	Protein family	Pathways	Functions
Q8IXM6; H0Y6T6; Q8IXM6-2	Nurim	NRM	Nurim (PTHR31040:SF1)		
Q14980-2; Q14980; A0A087WY61; Q14980-4; Q14980-3; Q14980-5	Nuclear mitotic apparatus protein 1	NUMA1	Nuclear mitotic apparatus protein 1 (PTHR18902:SF24)		
Q03405; Q03405-3; M0R0Y4; M0QYR6; M0R1I2; Q03405-2	Urokinase plasminogen activator surface receptor	PLAUR	Urokinase plasminogen activator surface receptor (PTHR10624:SF6)	Plasminogen activating cascade (P00050)/Blood coagulation (P00011)	
A2ACR1; P28065-2; P28065; A0A0G2JJA7; A2ACR0	Proteasome subunit beta type; Proteasome subunit beta type-9	PSMB9	Proteasome subunit beta type-9 (PTHR11599:SF50)	UPS	Ubiquitin proteasome system
X6R433; A0A0A0MT22; P08575-2; P08575; M3ZCP1; A0A075B788; E9PKH0	Protein-tyrosine-phosphatase; Receptor-type tyrosine-protein phosphatase C	PTPRC	Receptor-type tyrosine-protein phosphatase C (PTHR19134:SF284)	JAK/STAT signaling pathway (P00038)/B cell activation (P00010)/T cell activation (P00053)	T cell activation (P00053)
P05109	Protein S100-A8; Protein S100-A8, N-terminally processed	S100A8	Protein S100-A8 (PTHR11639:SF5)		Calmodulin signaling
P06702	Protein S100-A9	S100A9	Protein S100-A9 (PTHR11639:SF79)		Calmodulin signaling
P01011; G3V595; G3V3A0	Alpha-1-antichymotrypsin; Alpha-1-antichymotrypsin His-Pro-less	SERPINA3	Alpha-1-antichymotrypsin (PTHR11461:SF145)		Proteolysis (inhibitor)
A0A0C4DFU2; P04179; P04179-4; F5H4R2; A0A0C4DFU1; F5GYZ5; P04179-2; F5H3C5; G8JLJ2; A0A0C4DG56; P04179-3	Superoxide dismutase;Superoxide dismutase [Mn], mitochondrial	SOD2	Superoxide dismutase [Mn], mitochondrial (PTHR11404:SF6)		Antioxidant defense activity

essential to regulate T- and B-cell antigen receptor signaling by direct interaction with antigen receptor complexes or by activating Src family kinases (Pike and Tremblay, 2013). PTPRC also regulates cytokine receptor signaling by suppressing JAK kinase (Porcu et al., 2012). It has been reported that PTPRC is dysregulated in human miscarriage (Lorenzi et al., 2012). Increased levels of PTPRC in the fetal membranes of sPTB indicated activation of T- and B-cell antigen receptor signaling and suggested that sPTB may share a common pathophysiological mechanism with miscarriage.

Macrophage activation (CAPG, CD14, and CD163): CAPG is a member of the gelsolin/villin family of actin-regulatory proteins. CAPG reversibly blocks the barbed ends of F-actin filaments in a Ca^{2+}-dependent manner, thus capping the barbed ends of actin filaments and controlling actin-based motility of macrophages (Young et al., 1994). CD14 is a surface protein preferentially expressed on macrophages or monocytes. It mediates the innate immune response to bacterial lipopolysaccharide (Mogensen, 2009). A study in an animal model indicated that increased expression of TLR2 and CD14 was correlated with urea plasma parvum–induced fetal inflammatory response syndrome-like pathology (Allam et al., 2014). CD163 is a member of the scavenger receptor cysteine-rich superfamily and functions as an innate immune sensor for bacteria and an inducer of local inflammation (Fabriek et al., 2009). High levels of CD163 are associated with an increased risk of preterm delivery in pregnant women (Vogel et al., 2005). The detection of CAPG, CD14, and CD163 suggested the activation of macrophage or monocyte, even though we do not know what pathogen causes are.

Extracellular Matrix, Proteolysis, and Cell Adhesion

COL4A1, LAMA2, FBLN2, and APMAP are the ECM proteins. COL4A1 is an integral component of all basement membranes; it not only provides structural support, regulating adhesion, migration, and survival of cells, but also plays a key role in early placentation by modeling trophoblast cell invasion to remodel maternal spiral arteries and ensure sufficient blood flow to the developing fetus (Oefner et al., 2015). LAMA2 is a major component of the basement membrane and mediates the attachment and migration of cells into tissues during embryonic development by interacting with other ECMs. FBLN2 is an ECM protein belonging to the fibulin family. APMAP is a novel regulator of ECM components that may serve as a potential target to mitigate obesity-associated insulin resistance (Pessentheiner et al., 2017). The exact functions of LAMA2, FBLN2, and APMAP in the placenta are not clear; the expression level changes may indicate ECM

TABLE 2b | Proteins identified from fetal membranes associated with preterm birth (AB vs. CD): Down-regulated.

Majority protein IDs	Protein names	Gene names	Protein family	Pathways	Functions
Q15067-2; Q15067; Q15067-3	Peroxisomal acyl-coenzyme A oxidase 1	ACOX1	Peroxisomal acyl-coenzyme A oxidase 1 (PTHR10909:SF290)	Fatty acid metabolism	Fatty acid beta-oxidation
095573	Long-chain-fatty-acid–CoA ligase 3	ACSL3	Long-chain-fatty-acid–CoA ligase 3 (PTHR43272:SF13)	Fatty acid metabolism	Fatty acid metabolic process
P09525; Q6P452	Annexin A4; Annexin	ANXA4	Annexin A4 (PTHR10502:SF28)		
P40121; P40121-2; E7ENU9	Macrophage-capping protein	CAPG	Macrophage-capping protein (PTHR11977:SF13)	FAS signaling pathway (P00020)	Macrophage function/Structual molecule activity
P21291; E9PS42; E9PND2; E9PP21	Cysteine and glycine-rich protein 1	CSRP1	Cysteine and glycine-rich protein 1 (PTHR24215:SF23)		Structual molecule activity
Q9UHQ9; H7C0R7	NADH-cytochrome b5 reductase 1	CYB5R1	NADH-cytochrome b5 reductase 1 (PTHR19370:SF74)		Oxidoreductase activity
P05108; P05108-2; E7EPP8	Cholesterol side-chain cleavage enzyme, mitochondrial	CYP11A1	Cholesterol side-chain cleavage enzyme, mitochondrial (PTHR24279:SF3)	Androgen/estrogene/ progesterone biosynthesis (P02727)	
E7EQR4; P15311	Ezrin	EZR	Ezrin (PTHR23281:SF13)		Structual molecule activity
P15428; P15428-5; P15428-2; E9PBZ2; P15428-4	15-hydroxyprostaglandin dehydrogenase [NAD(+)]	HPGD	15-hydroxyprostaglandin dehydrogenase [NAD(+)] (PTHR44229:SF4)		
P40925; P40925-3; B9A041; P40925-2; B8ZZ51	Malate dehydrogenase, cytoplasmic	MDH1	Malate dehydrogenase, cytoplasmic (PTHR23382:SF3)	TCA cycle (P00051)	
E9PIY1; A0A0C4DGG1; Q9UKS6; E9PJ75; E9PNM9	Protein kinase C and casein kinase substrate in neurons protein 3	PACSIN3	Protein kinase C and casein kinase substrate in neurons protein 3 (PTHR23065:SF18)		
P30086	Phosphatidylethanolamine-binding protein 1; Hippocampal cholinergic neurostimulating peptide	PEBP1	Phosphatidylethanolamine-binding protein 1 (PTHR11362:SF28)	EGF/FGF receptor signaling pathway (P00018)	
P14618; P14618-3; B4DNK4	Pyruvate kinase PKM; Pyruvate kinase	PKM	Pyruvate kinase PKM (PTHR11817:SF15)	Glycolysis (P00024)	
P30044-2; P30044; P30044-3; P30044-4	Peroxiredoxin-5, mitochondrial	PRDX5	Peroxiredoxin-5, mitochondrial (PTHR10430:SF16)		
P61313; E7EQV9; E7ENU7; E7EX53; P61313-2	60S ribosomal protein L15; Ribosomal protein L15	RPL15	60S ribosomal protein L15 (PTHR11847:SF13)		
P62888; E5RI99; A0A0B4J213; A0A0C4DH44	60S ribosomal protein L30	RPL30	60S ribosomal protein L30 (PTHR11449:SF1)		Biosynthetic process
P46777	60S ribosomal protein L5	RPL5	60S ribosomal protein L5 (PTHR23410:SF12)		Biosynthetic process
P62277; J3KMX5	40S ribosomal protein S13	RPS13	40S ribosomal protein S13 (PTHR11885:SF6)		Biosynthetic process
P62249; M0R210; A0A087WZ27; M0R3H0; M0R1M5	40S ribosomal protein S16	RPS16;ZNF90			Biosynthetic process

TABLE 3 | Top-20 proteins identified from fetal membranes associated with preterm birth.

Gene symbol	Protein name	Function	*p*-value	Fold change
CSRP1	Cysteine and glycine-rich protein 1	Extracellular metrix	1.75E-03	−4.11
HPGD	15-hydroxyprostaglandin dehydrogenase [NAD (+)]	Inflammation	1.70E-03	−3.95
PKM	Pyruvate kinase	Glycolysis	1.46E-03	−3.59
ACSL3	Long-chain-fatty-acid-CoA ligase 3	Fatty acid metabolism	1.45E-03	−3.45
FBLN2	Fibulin-2	Extracellular metrix	1.36E-03	2.81
HMGN2	High mobility group nucleosomal binding domain 2	Gene transcription	2.19E-03	3.05
HMGN3	High mobility group nucleosomal binding domain 3	Gene transcription	2.19E-03	3.05
CD14	Monocyte differentiation antigen CD14	Macrophage function	1.49E-05	3.14
BST1	ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2	B-cell growth	1.92E-03	3.25
CD163	Scavenger receptor cysteine-rich type 1 protein M130	Macrophage function	8.10E-04	3.29
LMNB1	Lamin-B1	Extracellular metrix	6.31E-05	3.47
ICAM1	Intercellular adhesion molecule 1	Cell adhesion	5.86E-04	6.07
PTPRC	Receptor-type tyrosine-protein phosphatase C	T cell activation	5.36E-05	11.24
CYBB	Cytochrome b-245 heavy chain	Energy metabolism	7.48E-05	11.24
S100A9	Protein S100-A9	Calmodulin signaling	4.82E-03	11.47
MPO	Myeloperoxidase	Oxidative stress	5.18E-03	11.99
CTSG	Cathepsin G	Protein degradation	1.22E-03	13.56
ELANE	Neutrophil elastase	Protein degradation	6.02E-03	20.22
ITGAM	Integrin alpha-M	Cell adhesion	7.61E-05	28.71
MMP9	Matrix metalloproteinase-9	Protein degradation	5.25E-03	318.64

TABLE 4 | Proteins involved in top-scored pathways in fetal membrane of sPTB.

Pathway	Up-regulated protein	Down-regulated protein
Infection and inflammation	PTPRC, BST1, CAPG, CD14, CD163, S100A9	HPGD, S100P
Protein degradation and proteolysis	CTSG, ELANE, MMP9	
Extracellular matrix	APMAP, COL4A1, LAMA2, LMNB1, LMNB2, FBLN2	
Cell adhesion	ICAM1, ITGAM	CSRP1
Antioxidant	MPO	
Glycolysis		PKM
Fatty acid beta-oxidation		ACOX1, ACSL3

degradation in PPROM and PROM. CSRP1 is a membrane of the CRP family, which may regulate cellular development and differentiation.

MMP9, ELANE, and chymotrypsin C (CTSG) are the proteins involved in proteolysis. MMP9 has been reported to break down the ECM, such as type IV and V collagens. MMP9 is mainly expressed in amnion epithelia, chorion leave trophoblast, decidua parietalis, and placental syncytiotrophoblasts. The expression level of MMP9 was increased in fetal membranes from preterm and term labor as compared to non-labors (Xu et al., 2002). Moreover, fetuses with PPROM have higher concentrations of MMP-9 than those with preterm labor with intact membrane, indicating the pathogenic role of MMP-9 during a rupture of fetal membrane in sPTB (Romero et al., 2002). The level of MMP-9 has been used as a risk factor for preterm births (Di Ferdinando et al., 2010; Sorokin, 2010). In agreement with published data, an increase in MMP-9 expression may contribute to degradation of the ECM in the fetal membrane and in placentas, thus initiating sPTB. ELANE, a multifunctional serine protease stored in azurophilic granules of mature neutrophils, is able to degrade the ECM of connective tissue during an inflammatory process. It has been implicated that PPROM, microbial invasion of the amniotic cavity, and parturition at term and preterm are associated with a significant increase in the concentration of ELANE in the amniotic fluid. Another study suggested that ELANE levels in amniotic fluid may serve as a useful marker for predicting the duration of continued pregnancy after cervical cerclage (Hatakeyama et al., 2016). CTSG is a member of the peptidase S1 protein family in azurophilic granules of neutrophilic polymorphonuclear leukocytes. CTSG has a cleavage specificity similar to chymotrypsin C and may involve connective tissue remodeling at the site of inflammation. It has been shown that intra-amniotic inflammation (IAI) is associated with increased CTSG concentration in the amniotic fluid in PPROM (Musilova et al., 2017). It has been reported that the ubiquitin–proteasome–collagen (CUP) pathway is involved in collagen degradation in PPROM (Zhao et al., 2017). In addition, the CUP pathway was epigenetically regulated by IncRNA in PROM and PPROM (Luo et al., 2015). Even though we did not identify proteins belonging to the CUP pathway, we did identify the most significant proteolytic enzymes associated with PPROM and PTB, mainly MMP-9, serine protease (ELANE), and CTSG.

Our proteome data detected over-expression of cell adhesion molecules ICAM and ITGAM in sPTB. ICAM-1 is a cell

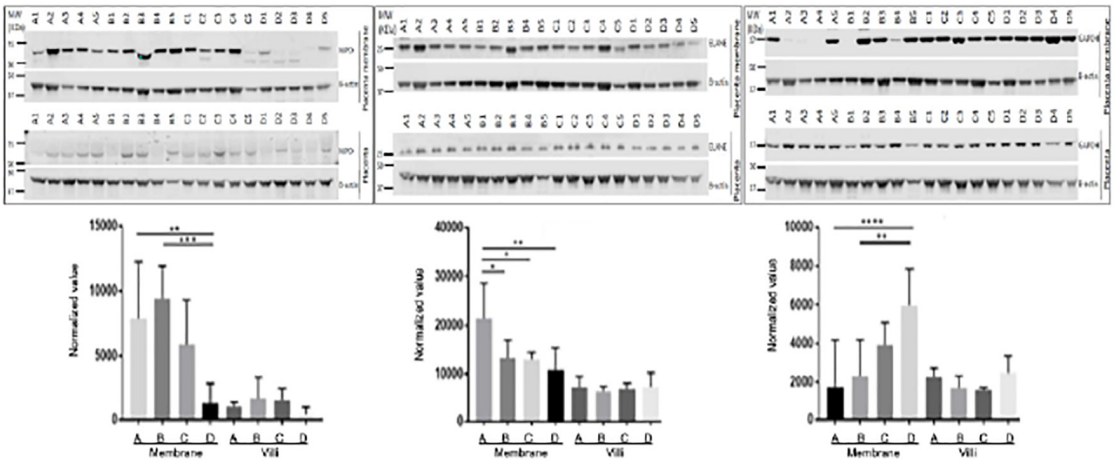

FIGURE 3 | Validation of differentially expressed proteins with placenta villi and fetal membranes. Randomly selected proteins MPO **(left panel)**, ELANE **(middle panel)**, and GAPDH **(right panel)** that were identified to be differentially expressed in fetal membrane (FM) vs. placental villi (PV) by MS/MS were validated with Western blots. Generally, three proteins were up-regulated in fetal membranes when compared to placental villi. MPO and ELANE were significantly increased in preterm pregnancies of sPTL (A) & PPROM (B) compared to full-term pregnancies of FTB (C) and PROM (D) fetal membranes. However, GAPDH was decreased in A + B. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$, and $****p < 0.0001$. Beta-Actin was used as an internal control for normalizing the protein expression value.

surface glycoprotein expressed on endothelial cells and cells of the immune system (Egal et al., 2014). It has been reported that ICAM-1 was overexpressed in villous trophoblasts during placental infection (Juliano et al., 2006). Another study indicated that expression of ICAM-1 by the human choriodecidua was elevated with preterm birth, together with increased leukocyte infiltration (Marvin et al., 1999). ITGAM plays an important role in the adherence of neutrophils and monocytes to stimulated endothelium. The level of ITGAM was significantly elevated in the amniotic fluid of women with preterm labor with IAI compared without IAI (Romero et al., 2010). Again, increased levels of ICAM-1 and ITGAM suggest activation of white blood cells and immune response in preterm birth placenta membrane.

Oxidative Stress Proteins MPO, LMNB1, and LMNB2

MPO is a heme protein synthesized and released from activated monocytes and neutrophils. Traditionally, it was described as a microbicidal enzyme. New evidence indicates that MPO generates hypochlorite-modified proteins, activates metalloproteinase (Prokopenko et al., 2002), and oxidatively consumes endothelium-derived nitric oxide in humans during normal pregnancy as well as during pathophysiologic processes (pre-eclampsia) (Hammer et al., 2001; Gandley et al., 2008). Higher cord blood levels of MPO are associated with preterm delivery (Yang et al., 2004). LMNB1 and LMNB2 are B-type nuclear lamin located in the inner nuclear membrane and play an important role in nuclear stability, chromatin structure, and gene expression. Overexpression of LMNB1 was found through a mitochondrial reactive oxygen species (ROS) *in vitro* model to increase the proliferation rate and to delay the onset of senescence (Shimi et al., 2011). Oxidative stress was higher and antioxidant enzymes were lower in PPROM compared with sPTB (Dutta et al., 2016). Our data suggest a higher level of oxidative stress, which may induce premature cellular senescence, inflammation, and proteolysis, eventually leading to membrane rupture and PPROM.

Glycolysis, Fatty Acid Synthesis

Differentially expressed proteins involved in glycolysis and FA synthesis were down-regulated in the fetal membranes of sPTB (**Table 3**). PKM, a catalytic enzyme involved in the last step of glycolysis, is responsible for dephosphorylating phosphoenolpyruvate to pyruvate and producing ATP under hypoxic conditions. PKM is also involved in angiogenesis in embryo development. It has been reported that the expression of PKM is higher in pre-eclampsia at delivery than in normal pregnancy (Bahr et al., 2014). ADPGK is a rate-limiting enzyme within the first step of glycolysis; it catalyzes the phosphorylation of D-glucose to D-glucose 6-phosphate by using ADP as the phosphate donor. GAPDH is an enzyme responsible for glyceraldehyde dehydration in the process of glycolysis. The placenta is a main source of high lactate levels during gestation. In later gestation, the concentration of lactate can reach 10 mmol/l, as compared to 1–2 mmol/l in the newborn and 0.5 mmol/l in maternal plasma. Fatty acids are essential substances for the construction of cell membrane and development of the nervous system. The disruption of FAs' metabolism in the maternal-placental interface would result in malnutrition of the fetus and in preterm birth (Bobinski and Mikulska, 2015). Blood levels of FAs are 20 times lower in the fetal circulation than in newborns' circulation (Makinde et al., 1998). This indicates that glucose/lactate provides the main energy source in fetal development. Typically, lactate is described as a waste product catalyzed by LDH under anaerobic metabolism. A metabolism study in various cell types indicated a model whereby lactate

generated in the cytosol compartment can be oxidized into pyruvate in mitochondria by LDH; pyruvate is subsequently transported into the inner membrane of the matrix and is then oxidized to acetyl CoA by pyruvate dehydrogenate (Kane, 2014). Acetyl CoA can then be fed into the TCA cycle and maintains mitochondrial function. Our data showed an increase in the level of ADPGK in the first step of glycolysis and a reduced level of PKM in the last step of glycolysis, suggesting an imbalance of glycolysis and the generation of less pyruvate through the conventional glycolysis pathway. To maintain metabolic functionality, a lactate oxidation process in mitochondria to produce pyruvate to sustain the TCA cycle is preferred. Reduction of the proteins involved in glycolysis and fatty acid synthesis may suggest that energy synthesis might be reduced in the fetal membrane tissues in sPTB. To confirm this, further investigation is needed to understand better the role of lactate's and FAs' metabolism in sPTB.

Tissue Specificity

Differential expression patterns of GAPDH were documented between the fetal membranes and placental villi. As shown in the right panel of **Figure 3**, GAPDH was found to be down-regulated in premature birth groups A and B, as compared to full-term control groups C and D with (PROM) or without (FTB) rupture of membrane in samples of fetal membrane. This finding is in agreement with the results generated from a discovery study with MS/MS. However, in the tissue of placental villi, Western blot for validation showed up-regulation, compared to FTB, which resulted in no significant change in placenta specimens between A + B vs. C + D. GAPDH is an enzyme involved in anaerobic energy metabolism through glycolysis. We speculated that the glycolysis pathway may be affected in fetal membrane and weakened the structure of fetal membrane but not in placental villi. To confirm this, further investigation should be performed to provide biochemical evidence.

CONCLUSION

By applying a proteomic approach, along with validation of proteomic results with Western blots, to study sPTL and PPROM with the capacity of distinguishing between the sPTL and PPROM groups, and of distinguishing the premature groups sPTL and PPROM from the mature groups FTB and PROM, we demonstrated a unique signature for each of the conditions, which is the strength this study. In addition, our proteomic data provide systemic insights into pathophysiological changes in the fetal membranes on the molecular level. Our data support the theory of inflammation/infection in sPTL and PPROM, even though we have not identified the source of infection. Inflammation increased the oxidative stress–triggered pyrolytic process that changes ECM structure, eventually rupturing the placental membrane and resulting in preterm birth. Metabolic function was also altered, in particular, imbalanced glycolysis and unconventional lactate oxidation, which may be associated with preterm birth. We are also aware of the limitation that applying the proteomic approach to identifying the differentially expressed protein(s) that are associated with clinical features and to identify biomarker(s) for the disease condition could be influenced by post-translational modification. Therefore, a larger sample size will need to be used for validation. In fact, studies on the inflammation-triggered proteolysis of ECM has been undertaken to verify a key pathogenic pathway for sPTB.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The study was approved by the Hospital Ethics Committee of Sanya Maternity and Child Care Hospital. Written informed consent was obtained from pregnant women to permit the use of placentas in research studies.

AUTHOR CONTRIBUTIONS

JP, XT, and HH contributed to the sample collection, processing and preparation, data acquisition, and laboratory work. NZ contributed to, and was responsible for, conceptual research design, initiating and coordinating the studies and experiments, data analysis, and drafting, finalizing, and submitting the manuscript. All authors contributed to the article and approved the submitted version.

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Progestins Inhibit Interleukin-1β-Induced Matrix Metalloproteinase 1 and Interleukin 8 Expression via the Glucocorticoid Receptor in Primary Human Amnion Mesenchymal Cells

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Preterm premature rupture of membranes is a leading cause of preterm births. Cytokine induced matrix metalloproteinase1 and interleukin 8 production from amnion mesenchymal cells may contribute to fetal membrane weakening and rupture. Progestins inhibit inflammation induced fetal membrane weakening but their effect on the inflammatory response of amnion mesenchymal cells is unknown. This study was designed to determine the role of progesterone receptor membrane component 1 and the glucocorticoid receptor in mediating the effects of progestins on interleukin-1β induced matrix metalloproteinase 1 and interleukin-8 expression in human amnion mesenchymal cells. Primary amnion mesenchymal cells harvested from human fetal membranes were passaged once and treated with vehicle, progesterone or medroxyprogesterone acetate at 10^{-6} M for 1 h followed by stimulation with interleukin-1β at 1 ng/ml for 24 h. Medroxyprogesterone acetate but not progesterone inhibited interleukin-1β-induced interlukin-8 and matrix metalloproteinase 1 mRNA expression. In subsequent dose response studies, medroxyprogesterone acetate, but not progesterone, at doses of 10^{-6}–10^{-8} M inhibited interleukin-1β induced interleukin-8 and matrix metalloproteinase 1 mRNA expression. We further demonstrated that inhibition of glucocorticoid receptor expression, but not progesterone receptor membrane component 1 knockdown with small interfering RNA transfection, resulted in a reversal in medroxyprogesterone acetate's (10^{-7} M) inhibition of interleukin-1β- induced matrix metalloproteinase 1 mRNA expression and interleukin-8 mRNA expression and protein expression. Our findings demonstrate that medroxyprogesterone acetate exerts its anti-inflammatory effect primarily through the glucocorticoid receptor in human amnion mesenchymal cells. Modulation of glucocorticoid receptor signaling pathways maybe a useful therapeutic strategy for preventing inflammation induced fetal membrane weakening leading to preterm premature rupture of membranes.

Keywords: progestins, preterm premature rupture of membranes, glucocorticoid receptor, progesterone receptor, interleukin-1 beta, Interleukin-8, matrix metalloproteinase 1

INTRODUCTION

Preterm birth (PTB) remains a major public health problem in the United States. Despite a slight decline in PTB rates from 2007 to 2014, rates have continued to increase in non-hispanic black women (Martin et al., 2017). Preterm births has multiple etiologies but the leading identifiable cause of preterm birth is preterm premature rupture of membranes (PPROM) (Parry and Strauss, 1998). Preterm premature rupture of membranes contributes significantly to perinatal morbidity and mortality, from adverse effects of prematurity and expectant management, increasing the risks of perinatal infections, placental abruption, umbilical cord prolapse, neonatal respiratory morbidity and adverse neurodevelopmental outcomes (Hadi et al., 1994; Lewis et al., 2007; Lee et al., 2010; Storness-Bliss et al., 2012; Korzeniewski et al., 2014; Ekin et al., 2015). Currently effective strategies for preventing PPROM are lacking.

The pathophysiology of PPROM involves the remodeling in fetal membranes of the extracellular matrix (ECM) in response to inflammation (Kumar et al., 2006). This inflammation induced ECM remodeling ultimately leads to fetal membrane weakening and rupture. *In vitro* biomechanical studies have also demonstrated that the amnion layer is the greatest contributor to the tensile strength of fetal membranes (Moore et al., 2006). The tensile strength of the amnion is due in part to the interstitial collagen type I and III in the compact layer of the amnion secreted by amnion mesenchymal cells in the fibroblast layer (Malak et al., 1993). Amnion mesenchymal cells are also a major source of matrix metalloproteinase 1 (MMP1) which initiates interstitial collagen degradation by cleaving the triple helix of the interstitial collagens (Mogami et al., 2013). Inflammatory cytokines induce MMP1 expression and activity in amnion mesenchymal cells which contributes to collagen degradation in the amnion ultimately leading to fetal membrane weakening and PPROM. Evidence suggesting that MMP1 plays a key role in PPROM include: elevated levels of MMP1 have been detected in the amniotic fluid of PPROM patients in both the presence and absence of infection (Maymon et al., 2000), a single nucleotide polymorphism in the promoter region of the MMP1 gene is associated with an increased risk of PPROM and changes in DNA methylation in the promotor region of the MMP1 gene have been associated with an increased risk of PPROM (Wang et al., 2008).

Our preliminary secretomic analysis of human amnion mesenchymal cells have demonstrated that amnion mesenchymal cells can release interleukin 8 (IL8) in response to interleukin-1 beta (IL1β) stimulation. interleukin 8 is a potent neutrophil chemoattractant and stimulator of neutrophil degranulation. Neutrophils in turn release MMP8 which cleaves the interstitial collagens. Neutrophil infiltration in fetal membranes has been associated with infection induced and abruption induced PPROM (Helmig et al., 2002; Lockwood et al., 2005). IL8 has also been implicated in epithelial to mesenchymal transition – a mechanism which has been implicated in the pathophysiology of PPROM (Radisky, 2005; Janzen et al., 2017). An increase in IL8 levels in amniotic fluid maybe associated with PPROM and predict the onset of preterm labor (Rizzo et al., 1997; Zhang et al., 2000; Jia, 2014). These findings collectively suggest that mesenchymal cells in response to inflammation play a role in the initiation of mechanism that lead to PPROM and PTB.

Progestins are used clinically for the prevention of PTB in women with a prior history of spontaneous PTB (Meis et al., 2003). *In vitro* studies have demonstrated that progestins are able to attenuate inflammation induced fetal membrane weakening (Kumar et al., 2015). The mechanisms by which progestins inhibit fetal membrane weakening still remains unclear. Given the role of the amnion mesenchymal cells in maintaining fetal membrane integrity, the effect of progestins on the inflammatory response of amnion mesenchymal cells may provide some insight into possible progestin-mediated mechanisms. Interestingly, fetal membranes do not express the classical nuclear progesterone receptors but still remain progesterone responsive and this progesterone responsiveness may be mediated through membrane-associated progesterone receptors (Merlino et al., 2009; Luo et al., 2010). For example, fetal membranes express progesterone receptor membrane component 1 (PGRMC1) whose role in fetal membranes remains to be elucidated (Feng et al., 2014, 2016; Allen et al., 2015). Furthermore, in fetal membranes, the amnion expresses higher levels of PGRMC1 when compared with the maternally derived decidual layer (Feng et al., 2014). We have previously demonstrated that PGRMC1 protein expression is diminished in PPROM patients when compared with term and preterm no labor patients highlighting the fact that PGRMC1 may play a role in molecular mechanisms that lead to fetal membrane rupture (Feng et al., 2014). Functionally we have shown that PGRMC1 partially mediates the inhibition of progestins on cytokine induced MMP9 activity in the HTR8 cytotrophoblast cell line and primary amnion epithelial cells (Allen et al., 2014, 2019). PGRMC1 may also play a role in oxidative stress induced senescence in fetal membranes (Feng et al., 2019). These findings demonstrate that PGRMC1 plays a role in maintaining fetal membrane integrity but its role in the amnion mesenchymal cells still remains unknown.

In the absence of the nuclear progesterone receptor, the glucocorticoid receptor (GR) may also explain some of the effects of progestins in fetal membranes. Glucocorticoids have been shown to inhibit lysyl oxidase (LOX) expression via the GR in amnion mesenchymal cells, a mechanism that may lead to fetal membrane rupture *in vivo* (Liu et al., 2016). Another study suggested that the inhibition of inflammation induced fetal membrane weakening *in vitro* by progestins could also be GR mediated (Kumar et al., 2015). Taken together our objectives were firstly to demonstrate that progestins inhibit IL1β-induced MMP1 and IL8 mRNA expression and secondly to determine if this mechanism is mediated through PGRMC1 or GR. Our primary hypothesis was that Progestins inhibit IL1β-induced MMP1 and IL8 mRNA expression primarily through PGRMC1.

MATERIALS AND METHODS

Isolation of Amnion Mesenchymal Cells

The collection of fetal membrane samples was approved by the Duke Medicine Institutional Review Board with a waiver of

consent. As a result, fetal membrane samples were deidentified and there was no link to any clinical information. Fetal membrane samples were collected from term healthy patients at elective cesarean section without prior rupture of membranes or labor using a previously described protocol with modifications (Casey and MacDonald, 1996). Briefly, the amnion was separated from the choriodecidua and rinsed three times in Dulbecco modified Eagle medium: Nutrient Mixture F12 (DIMEM/F12) (Thermo Fisher Scientific) media with penicillin, streptomycin and amphotericin-B (Anti/Anti) (Thermo Fisher Scientific). Amnion epithelial cells were released from the amnion which was minced using scalpel blades and then digested with 1 g of 1:250 trypsin in DIMEM/F12 media with Anti/Anti for 30 min at 37°C. The remaining undigested amnion was collected and washed in DIMEM/F12 with Anti/Anti after filtering using a metal strainer. The process was repeated two more times. The undigested tissue fragments from three digestions were then pooled and incubated in DIMEM/F12 with Anti/Anti containing 0.75 mg/ml of Type I collagenase at 37°C for 30 min to release the amnion mesenchymal cells. The isolated cells were collected after filtration of the remaining undigested tissue through a 70 μm cell strainer. The filtrate was centrifuged at 1000 *g* for 5 min and the cell pellet was re-suspended in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and plated in 10 cm culture dishes. The cell cultures were incubated in humidified air and 5% CO2 for 5–7 days until they achieved confluence. Cells were passaged only once using 0.25% trypsin with EDTA and plated at approximately 0.2–0.5 × 10^6 cells/ml for all subsequent experiments.

Treatments

Amnion mesenchymal cells were plated at 0.5 × 10^6 cells/ml for 24 h. To determine the effect of progestin therapy on IL1β-induced MMP1 and IL8 mRNA expression the cell cultures were pre-treated with ethanol, medroxyprogesterone acetate (MPA), or progesterone (P4) (Millipore Sigma) at 10^{-6} M for 1 h followed by stimulation with IL1β at 1 ng/ml (RnD systems) for 24 h in DIMEM/F12 with Anti-Anti and 1% FBS. Subsequent dose response studies were performed using doses of MPA and P4 ranging from 10^{-6} to 10^{-8} M. At the end of the experimental incubation, cell culture media was harvested and centrifuged at 12,000 *g* for 5 min and the supernatant was collected, aliquoted and frozen at −80°C. Trizol® lysates were harvested and frozen at −80°C.

PGRMC1 and GR Depletion With siRNA

To determine the effect of PGRMC1 and GR on progestin mediated inhibition of IL1β-induced MMP1 and IL8 mRNA levels amnion mesenchymal cells were depleted of PGRMC1 or GR using siRNA. In a separate series of experiments amnion mesenchymal cells were initially plated at 0.2–0.5 × 10^6 cells/ml for 24 h in DIMEM F12 with 10%FBS. The cultures were then transfected using Lipofectamine RNAiMax and 10 nmol of PGRMC1 siRNA (ID: S21310), GR siRNA (ID: AM513311) or control siRNA (ID: AM4611) for 24 h in both serum and antibiotic free media. After 24 h transfection, the cultures were supplemented with 1 ml of DIMEM/F12 with 20% serum and incubated for an additional 48 h. At the end of the 72 h incubation the cells were then pre-treated with MPA or P4 for 1 h followed by stimulation with or without IL1β 1 ng/ml in DMEM/F12 with Anti/Anti and 1% FBS for an additional 24 h. We assessed the efficacy of PGRMC1 and GR knockdown with siRNA when compared with the control siRNA group using both real-time PCR and Western Blot.

Quantitation of IL8 and MMP1 Protein Concentrations by Magnetic Luminex Assay

Interleukin-8 and MMP1 levels in cell culture media were quantified simultaneously using the Human Magnetic Luminex assay (RnD systems) as directed by the manufacturer's protocol. The range of quantitation for MMP1 was 49.8–13,520 pg/ml. The range for quantitation of IL8 was 5.2–1227 pg/ml. Cell culture supernatant samples were diluted 1:10 due to the high concentration of IL8 in these samples to allow measurement within the range of the assay. When IL8 levels were below the lower limit of quantitation, we reported 1/2 of the lower limit of quantitation for IL8. In contrast, MMP1 levels were significantly lower in cell culture media and in the diluted samples they were below the level of quantitation of the assay and were not reported.

RNA Isolation and Real Time Quantitative PCR

Total RNA was extracted from amnion mesenchymal cells using Trizol, isolated using the RNeasy Mini-Kit and RNA concentrations were quantified using the NanoDrop® spectrophotometer. For each sample, 0.5–1.0 μg of RNA was reversed transcribed into cDNA using the Superscript III® first strand system (Thermo Fisher Scientific). Twenty-five to fifty nanograms of cDNA were used as the template for each real-time PCR reaction. Real-time PCR was performed using pre-validated Taqman probes directed against *MMP1* (assay ID: Hs00899658_m1) and GR (*NR3C1*) (Assay ID:Hs00353740_m1). Forward and reverse primers were used to detect PGRMC1, IL8 mRNA and the housekeeping gene *B2M* mRNA expression (**Table 1**). We performed Real-Time PCR using the following protocol: initial denaturation at 95°C for 3 min, followed by a 2-step amplification process of 95°C for 30 s and 60°C for 40 s for a total of 40 cycles.

TABLE 1 | Primer sequences used for real-time quantitative PCR.

Gene	Primer sequence
IL8	Forward 5′-ACT GAG AGT GAT TGA GAG TGG AC-3′
	Reverse 5′-AAC CCT CTG CAC CCA GTT TTC-3′
PGRMC1	Forward 5′-TGT GAC CAA AGG CCG CAA AT-3′
	Reverse 5′-TGC TTC CTT ATC CAG GCA AAA T-3′
B2M	Forward 5′-GAG GCT ATC CAG CGT ACT CCA-3′
	Reverse 5′-CGG CAG GCA TAC TCA TCT TTT-3′

Real-Time PCR was performed using the iCycler IQ™ Real-Time PCR detection system (Bio-Rad). All samples were run in duplicates with the mean cycle threshold C_t for the gene of interest normalized to the mean C_t value for the housekeeping gene *B2M*.

Western Blot

At the end of each experiment, cell culture media was removed, and the cells were washed with ice cold PBS and then lysed with radioimmunoprecipitation (RIPA) buffer containing the Complete Mini® protease inhibitor cocktail (Millipore Sigma). Total protein content for each sample was quantified using the Bradford protein assay. An equal amount of protein (10 μg) were separated on a 4–12% Bis –Tris gels (Thermo Fisher Scientific) at 125 V for 60 min and transferred onto a polyvinylidene fluoride (PVDF) membrane. The PVDF membranes were blocked with 5% milk in tris-buffered saline with 0.01% Tween for 1 h and then were incubated with polyclonal rabbit anti-human PGRMC1 (1:2000, catalog No. HPA08277, Millipore Sigma), polyclonal rabbit anti-human GR (1:1000 catalog No. 3660S, Cell Signaling) or monoclonal rabbit anti-human B2M (1:10,000, Catalog No. 12851S, Cell Signaling) antibodies overnight at 4°C. The membranes were then incubated with the appropriate secondary antibody (horseradish peroxidase-linked anti-rabbit or anti-mouse IgG at 1:1000 dilution, Cell Signaling) for 1 h at room temperature, after which they were incubated with the SuperSignal® West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and exposed on X-ray films. The band densities were quantified using ImageJ® and both PGRMC1 and GR were normalized to B2M.

Immunofluorescence

Primary amnion mesenchymal cells were plated on chamber slides at 1×10^4 cell/ml in DIMEM/F12 with antibiotics-antimycotics and 10% FBS for 48 h. Ice cold methanol was then used to fix the cells at −20°C for 10 min. The cells were then incubated for 30 min with Image – IT™ Signal Enhancer (Thermo Fisher Scientific) after which they were permeabilized and blocked with 5% goat serum and 0.1% Triton™ X for 1 h. To localize PGRMC1 or GR cells were incubated with rabbit anti-human polyclonal PGRMC1 antibody 1:100 (catalog No. HPA08277, Millipore Sigma) and/or mouse anti-human monoclonal GR antibody 1:250 (catalog no. SAB4800041, Millipore Sigma) overnight at 4°C in a humidified slide chamber. Cells incubated with a monoclonal anti-mouse (catalog no. MA5-14453, Thermo Fisher Scientific) and polyclonal anti-rabbit antibody (catalog no. ab27472, Abcam) were used as negative controls. To determine the homogeneity of the culture the cells were stained with the mesenchymal cell marker mouse anti-human monoclonal vimentin antibody (clone v9, catalog no. M0725, Agilent Dako) at 1:200 dilution. The cells were then incubated for 1 h with the Alexa Fluor™ 488 goat anti-mouse ReadyProbes™ (Thermo Fisher Scientific) and/or Alexa Fluor™ 594 goat anti-rabbit ReadyProbes™ (Thermo Fisher Scientific) diluted based on the manufacturer's specifications. The cells were then incubated with DAPI 1:1000 for 5 min. ProLong™ Diamond Antifade (Thermo Fisher Scientific) was used as the mounting media and the cells were imaged with the Zeiss Axio Imager fluorescence microscope.

Statistical Analysis

All experimental groups were compared using one-way analysis of variance (ANOVA) with *post-hoc* pairwise comparisons using the Sidak test with each *P*-value was adjusted for multiple comparisons. A $p < 0.05$ was considered significant. Data were analyzed using GraphPad® Prism. Data are presented as mean ± standard error of the mean (sem).

RESULTS

Immunofluorescent staining demonstrated that PGRMC1 is localized to the nucleus, the perinuclear area, and the cytoplasm of amnion mesenchymal cells (**Figures 1B,E**). Interestingly, GR localized to the nucleus in some cells (**Figure 1A**) and in the cytoplasm in other cells (**Figure 1D**). When GR and PGRMC1 were both expressed in the nucleus they appeared to co-localize in the nucleus (**Figure 1C**). When GR was primarily expressed in the cytoplasm there was no evidence of co-localization with PGRMC1 (**Figure 1G**). The majority of the cells (>95%) stained positive for the mesenchymal cell marker vimentin (**Figure 1H**). Primary amnion mesenchymal cells were stained with DAPI to localize the nucleus (**Figure 1F**). The negative control demonstrated no evidence of non-specific staining (**Figure 1I**).

The Effect of Medroxyprogesterone Acetate and Progesterone on IL1β-Induced MMP1 and IL8 mRNA Expression in Amnion Mesenchymal Cells

Interleukin-1β significantly induced both *MMP1* and *IL8* mRNA levels in primary amnion mesenchymal cells when compared with the unstimulated (vehicle) control. In initial experiments MPA at a dose of 10^{-6} M significantly inhibited IL1β-induced *MMP1* and *IL8* mRNA expression when compared with the stimulated control (vehicle control plus IL1β) while P4 did not show any effects (**Figures 2A,B**). Both MPA and P4 did not suppress basal *MMP1* or *IL8* mRNA expression in amnion mesenchymal cells when compared with the unstimulated control.

In subsequent dose response studies pre-treatment with MPA at doses of 10^{-6}, 10^{-7}, and 10^{-8} M significantly inhibited IL1β-induced *MMP1* and *IL8* mRNA expression when compared with the stimulated controls (**Figures 2D,F**). Surprisingly, pre-treatment with P4 at doses of 10^{-7} and 10^{-8} M were associated with a significant increase in IL1β-induced *MMP1* mRNA expression when compared with the stimulated control (**Figure 2C**). Pre-treatment with all doses of P4 had no significant effect on IL1β-induced IL8 mRNA expression when compared with the stimulated control (**Figure 2E**). All doses of MPA and P4 tested had no effect on both basal *MMP1* and *IL8* mRNA expression when compared with the unstimulated control. In the

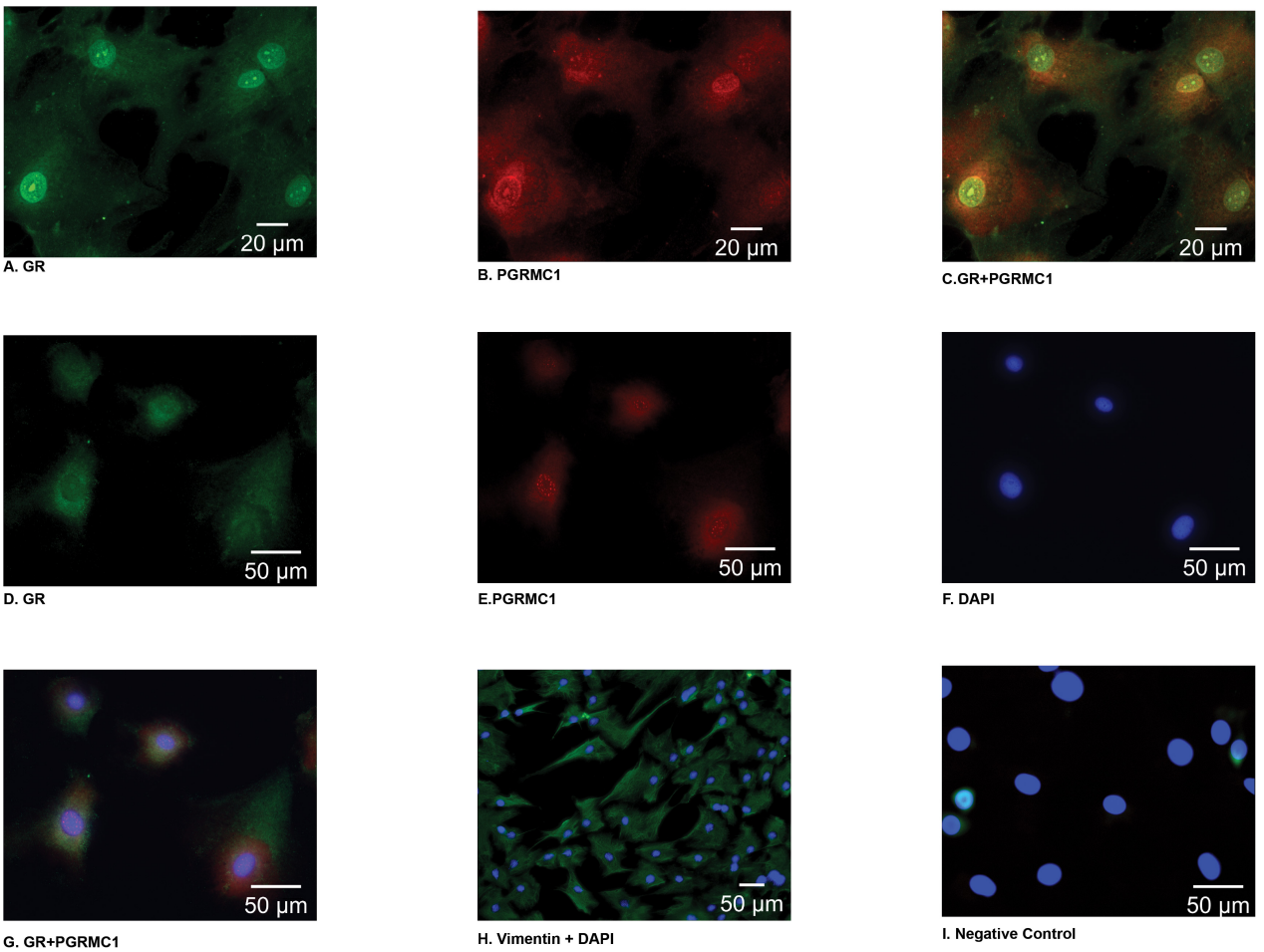

FIGURE 1 | In primary amnion mesenchymal cells GR was localized (green) primarily to the nucleus in some cells **(A)** and the cytoplasm in other cells **(D)**, PGRMC1 (red) localized to the nucleus and the perinuclear area of the cytoplasm **(B,E)**. Colocalization of GR and PGRMC1 in the nucleus when GR is expressed in the nucleus **(C)** vs. when GR is primarily expressed in the cytoplasm **(G)**. Primary mesenchymal cells stained with DAPI **(F)**, vimentin to identify primary mesenchymal cells **(H)**, and the negative control **(I)**. Images **(A–C)** were captured at 63× magnification **(D–G)**, **(I)** 40× magnification, and **(H)** at 20×.

subsequent siRNA experiments, we used MPA at a dose of 10^{-7} M and P4 at a dose of 10^{-6} M.

The Role of PGRMC1 and GR on Progestins Mediated Inhibition of IL1β-Induced MMP1 and IL8 Expression in Amnion Mesenchymal Cells

PGRMC1 siRNA significantly inhibited *PGRMC1* mRNA and protein expression in amnion mesenchymal cells but had no significant effect on GR mRNA and protein expression when compared with the control siRNA group (**Figure 3**). GR siRNA significantly inhibited GR mRNA and protein expression but had no significant effect on *PGRMC1* mRNA and protein expression when compared with the control siRNA group (**Figure 3**).

In the control siRNA group, the inhibition of IL1β-induced *MMP1* mRNA expression by MPA at 10^{-7} M when compared with the stimulated control was significantly attenuated by GR siRNA but was unaffected by PGRMC1 siRNA treatment (**Figure 4A**). As we had previously observed P4 at a dose of 10^{-6} M had no effect on IL1β-induced *MMP1* mRNA expression when compared with the stimulated control in the control siRNA group and this effect was unaffected by PGRMC1 or GR inhibition with siRNA. Furthermore, both MPA and P4 had no significant effect on basal MMP1 mRNA expression and this was unaffected by PGRMC1 and GR inhibition by siRNA.

In the control siRNA group, the inhibition of IL1β-induced *IL8* mRNA expression and IL8 protein levels in cell culture media by MPA at a dose of 10^{-7} M when compared with the stimulated control was significantly attenuated by GR inhibition with siRNA but was again unaffected by PGRMC1 inhibition with siRNA (**Figures 4B,C**). Progesterone at a dose of 10^{-6} M had no significant effect on both IL1β-induced IL8 mRNA expression and protein concentration when compared with the stimulated control in the control siRNA group and this lack of effect was unaffected by either PGRMC1 or GR inhibition. Both MPA and P4 had no significant effect on basal IL8 mRNA expression or protein concentration when compared with the

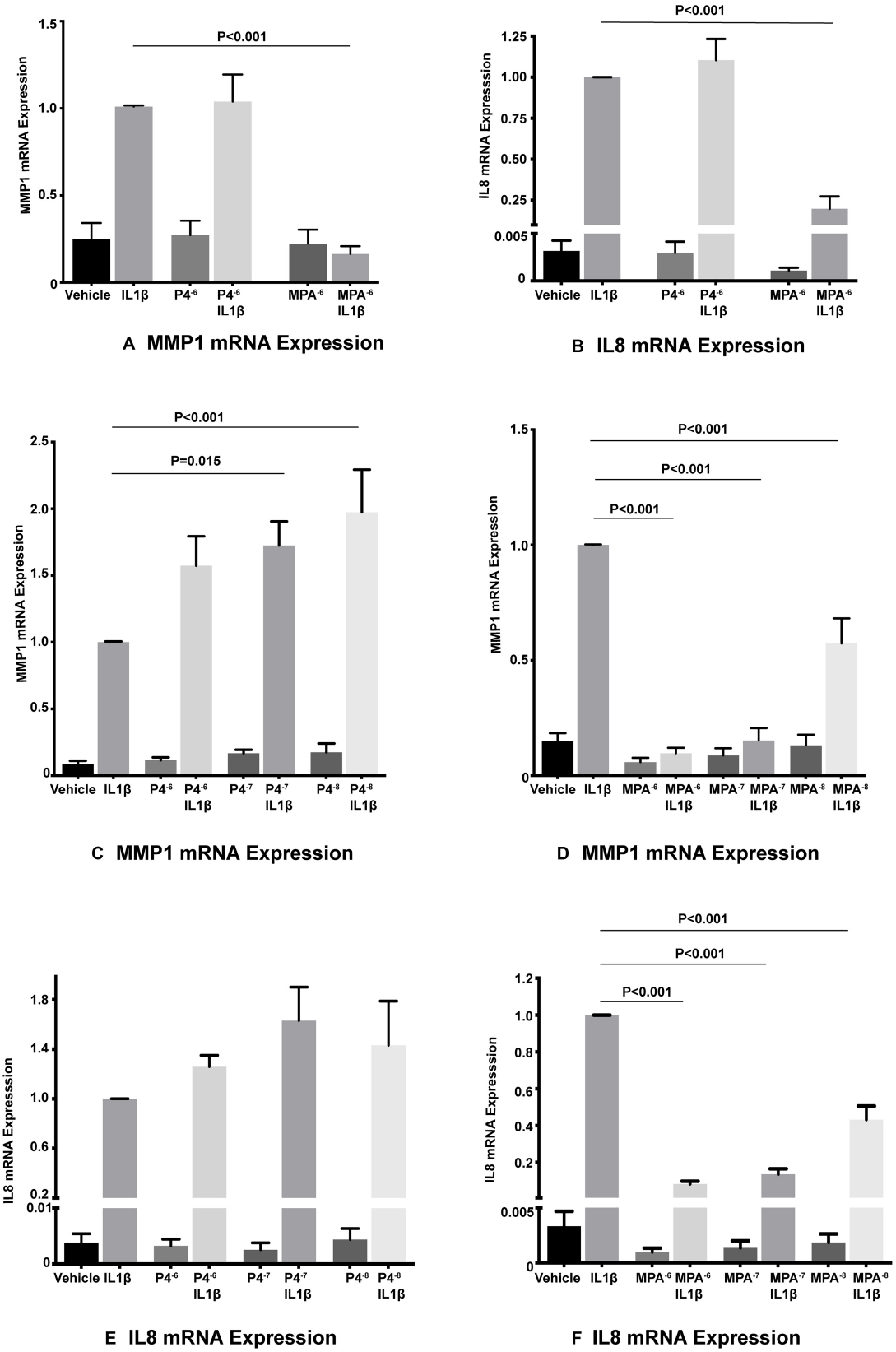

FIGURE 2 | MPA but not P4 inhibits IL1β-induced *MMP1* and *IL8* mRNA expression in primary amnion mesenchymal cells **(A,B)**. P4 dose response studies **(C,E)**, and MPA dose response studies **(D,F)** on IL1β-induced *MMP1* and *IL8* mRNA expression (n = 6–7 patients).

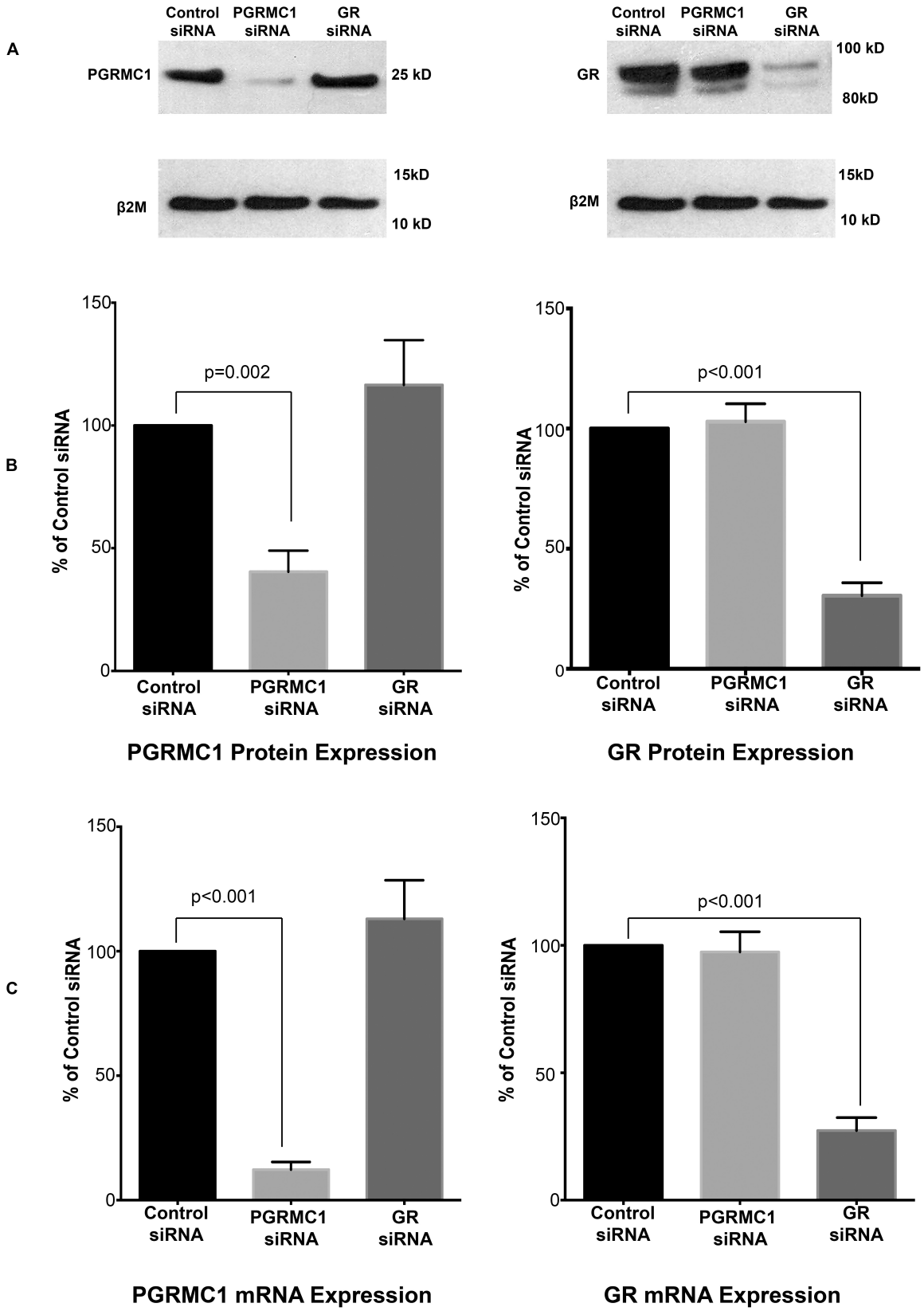

FIGURE 3 | PGRMC1 siRNA significantly inhibits PGRMC1 protein expression **(A,B)** and *PGRMC1* mRNA levels **(C)**. GR siRNA significantly inhibits GR protein expression **(A,B)** and GR mRNA levels **(C)** (n = 10 patients). For illustrative purposes the B2M image (top panel lower blot) was reused in both images.

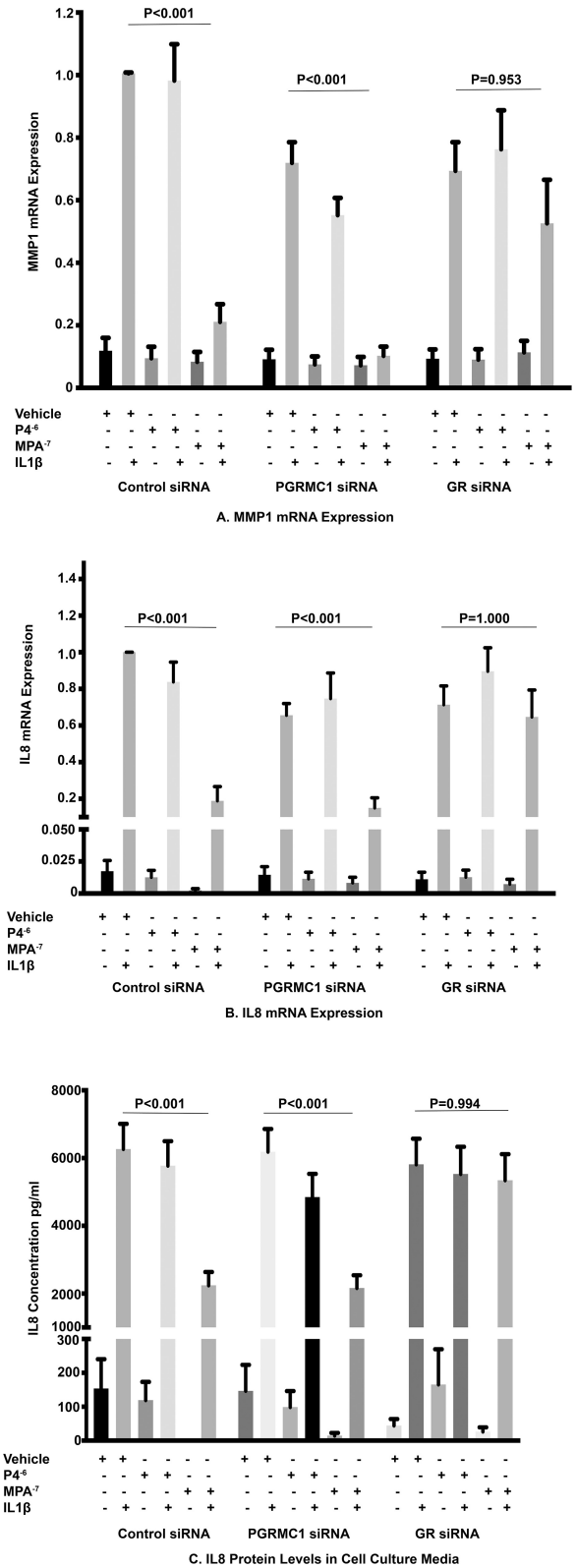

FIGURE 4 | The role of PGRMC1 siRNA and GR siRNA on IL1β-induced *MMP1* mRNA expression **(A)** and IL1β-induced *IL8* mRNA and protein levels **(B,C)** (n = 11 patients).

unstimulated control and this was unaffected by PGRMC1 and GR inhibition by siRNA.

DISCUSSION

Our findings demonstrate that MPA but not P4 inhibit IL1β-induced MMP1 mRNA expression and IL8 mRNA levels and secreted protein levels through GR and not through PGRMC1 in amnion mesenchymal cells. These findings are similar to our previous work in amnion epithelial cells which demonstrated that the inhibition of cytokine-induced MMP9 activity and mRNA levels in amnion epithelial cells was mediated through GR (Allen et al., 2019). Additionally, PGRMC1 localizes to the perinuclear area and the nucleus which is stark contrast to its expression pattern in amnion epithelial cells where it primarily localized to the cytoplasm and perinuclear area (Allen et al., 2019). GR in turn localizes to both the nucleus and cytoplasm as has been previously described (Wikström et al., 1987). Since in the inactive state, GR is localized to the cytoplasm as a part of a multiprotein complex with chaperone proteins and immunophilins, localization to the nucleus could represent activation by ligands present in cell culture media (Matthews et al., 2011). Alternatively the heterogeneity of GR localization in amnion mesenchymal cells could represent ligand independent trafficking of GR between the nucleus and cytoplasm which may occur normally during different phases of the cell cycle (Matthews et al., 2011). Both proteins appear to co-localize to the nucleus and even though the clinical relevance of this co-localization remains unclear, it could represent a functional interaction between both receptors in regulating genes involved in inflammation, cell cycle regulation and apoptosis in amnion mesenchymal cells and fetal membranes (Peluso et al., 2010; Allen et al., 2014, 2019; Sueldo et al., 2015).

This study provides further evidence that the anti-inflammatory effects of progestins, specifically MPA in the amnion are primarily mediated through GR and this is particularly important in fetally derived cells that lack the nuclear progesterone receptor (Merlino et al., 2009; Allen et al., 2014). The findings are even more important given the central role that amnion mesenchymal cells play in collagen turnover and immunomodulation in fetal membranes (Casey and MacDonald, 1996). The classic glucocorticoid receptor GRα, which is ubiquitously expressed, is known to mediate most of the known biological effects of glucocorticoids. However, there is still sparse data on the expression patterns of GR in fetal membranes in the preterm delivery phenotypes. This is further complicated by the fact that alternative mRNA splicing and translation initiation sites leads to the generation of multiple GR isoforms (Lu and Cidlowski, 2005; Lu et al., 2007; Turner et al., 2007). Currently, at least 8 GR isoforms have been identified in the placenta and are affected by gestational age and fetal sex and the roles of these isoforms still remain unclear (Saif et al., 2015). GRα-A is one of the isoforms involved in mediating glucocorticoid effects through its ability to transcriptionally activate and repress multiple gene targets. Interestingly the relative expression of GRα-A in the nucleus is less in preterm placentae than term placentae (Saif et al., 2015). The GR isoforms

in the fetal membranes remain unknown, however, identification of the isoforms which mediate anti-inflammatory effects in fetal membranes could allow the development of safer glucocorticoids with reduced side effects.

Our findings in this study highlight the need for elucidating the underlying mechanism by which GR exerts these effects in fetal membranes. Recently it has been determined that distinct negative glucocorticoid response elements (nGRE) mediate the transcriptional repression effects of GR via an inverted quadrimetric palindrome separated by 0–2 nucleotide pairs (Surjit et al., 2011). Alternatively the protein-protein interaction between GR and specific transcription factors at promoters can result in inhibition (or stimulation) of target genes. These promoters either do not contain GRE (tethering) or have both GREs and responsive elements for the transcription factors that associate with GR (composite promoters) (Reichardt et al., 1998). Transcription factors that have been implicated in this protein-protein interaction include NF-κB, AP-1, and STATs (Heck et al., 1994; Stöcklin et al., 1996; De Bosscher et al., 1997). Interestingly NF-κB, AP-1 and STATs are some of the key transcription factors involved in the transcriptional regulation of MMP1 and IL8 gene expression (Chaudhary and Avioli, 1996; Overall and López-Otín, 2002; Fanjul-Fernández et al., 2010; Lin et al., 2016). Elucidating the anti-inflammatory mechanisms of GR in the amnion mesenchymal cells may allow the development of tissue specific GR modulators which inhibit inflammatory induced fetal membrane weakening and PPROM.

The role of PGRMC1 also remains unclear in fetal membranes. PGRMC1 did not mediate MPA's anti-inflammatory effect and we were also unable to demonstrate an anti-inflammatory effect of progesterone on IL1β-induced inflammation in amnion mesenchymal cells. However, emerging evidence demonstrates that PGRMC1's effects maybe cell type specific. In our previous work we demonstrated that PGRMC1 partially mediated the inhibition of TNFα-induced MMP9 activity by MPA in HTR8 cells, a cytotrophoblast cell line and to a lesser extent in primary human amnion epithelial cells (Allen et al., 2014, 2019). More recently we have demonstrated that PGRMC1 plays a role in mediating oxidative stress induced cellular aging through p38 MAPK and SIRT3 in primary human chorion cells (Feng et al., 2019). It has also been demonstrated that PGRMC1 may have anti-inflammatory effects by suppressing TNFα-induced gene expression independent of progesterone in N42 hypothalamic cells (Intlekofer et al., 2019). While in this study we were unable to demonstrate that PGRMC1 plays a role in IL1β-induced inflammation in amnion mesenchymal cells, it is likely that it may regulate other pathophysiological pathways that lead to PPROM and therefore warrants further investigation.

Interestingly in our *in vitro* study P4 was ineffective in preventing IL1β-induced MMP1 and IL8 expression and lower doses of P4 were associated with increased IL1β-induced MMP1 mRNA expression. In our prior work we have also been unable to demonstrate that P4 effectively prevents cytokine induced MMP9 activity and mRNA expression in primary amnion epithelial and chorion cells (Allen et al., 2015). The lack of effect of P4 and its augmentation of IL1β-induced MMP1 mRNA expression at lower doses could partly be explained by the lack of expression of the nuclear progesterone receptor in amnion mesenchymal cells and modulatory effects mediated via GR, respectively. The nuclear progesterone receptor isotypes PR-A and PR-B mediate most of the anti-inflammatory actions of P4 (Patel et al., 2014). In fact PR-A and PR-B null female mice demonstrate marked inflammatory changes in the endometrium (Lydon et al., 1995). Therefore in the absence of the nuclear progesterone receptor, P4's anti-inflammatory effects maybe significantly attenuated. However, P4 also binds GR but it does so with low relative affinity and it may also act as a weak partial agonist for GR-mediated transactivation and transrepresion (Fuhrmann et al., 1996; Koubovec et al., 2005; Africander et al., 2011). Therefore the augmentation of IL1β-induced MMP1 mRNA expression could represent dose dependent conformational changes leading to GR-mediated transactivation and expression of proinflammatory genes. These anti and proinflammatory GR mediated effects highlight the complexity of GR signaling and the importance of finding the middle ground in maximizing GR-mediated therapeutic benefits.

Significant controversy surrounds the clinical use of progesterone for PTB prevention. Preclinical studies have demonstrated that progesterone promotes uterine quiescence by suppressing the expression of contraction associated proteins, inhibiting the expression of proinflammatory chemokines and cytokines and inhibiting immune cell infiltration and activation in the myometrium, potentially preventing mechanisms that may lead to PTB (Lei et al., 2015; Nadeem et al., 2016; Edey et al., 2017; Amini et al., 2019). In the cervix functional progesterone withdrawal is also associated with a local increase in proinflammatory mediators, matrix metalloproteinases and increased recruitment of immune cells that induces cervical remodeling that leads to PTB in human and animal models (Denison et al., 2000; Kuon et al., 2010; Kirby et al., 2016). While these findings suggest that progesterone supplementation maybe a useful therapeutic intervention for PTB prevention at least three large clinical trials have now demonstrated that vaginal progesterone does not significantly reduce preterm birth rates and in 2 of the studies, it did not reduce the rates of PPROM in subgroup analyses (O'Brien et al., 2007; Norman et al., 2016; Crowther et al., 2017; Norman and Bennett, 2017). The most recent PROLONG trial also demonstrated that another progestin 17αhydroxyprogesterone acetate did not significantly reduce recurrent spontaneous PTB (Blackwell et al., 2020). This has prompted some researchers to opine that it is now time to examine alternative therapies to progesterone for PTB prevention (Norman and Bennett, 2017). However, given the multiple mechanisms that may lead to PTB, research now needs to be focused on identifying the patient populations that may derive benefit from progesterone therapy.

Our study has several limitations. Firstly, we were unable to quantify MMP1 protein levels in part because the samples had to be diluted for IL8 level quantification using the magnetic Luminex assay. Another limitation of the study is that we only investigated inflammation induced molecular pathways, so our findings may not apply to other initiators of PPROM such as thrombin. Thrombin also induces *MMP1* mRNA expression, IL1β and IL8

protein levels in human amnion mesenchymal cells (Chigusa et al., 2016). In amnion mesenchymal cells this inflammatory response to thrombin can be inhibited by activators of nuclear factor erythroid 2- related factor 2 (NRF2) a transcription factor that mediates the expression of cell defense and antioxidant genes (Chigusa et al., 2016). Recently it has been demonstrated that GR signaling may also modulate NRF2 transcriptional activity, potentially highlighting the central role GR may play in pathways leading to PPROM and PTB (Alam et al., 2017).

In summary our findings provide additional evidence that the progestin MPA exerts its anti-inflammatory effects on molecular pathways implicated in PPROM through GR and not through PGRMC1 in fetal membranes. Identifying the downstream mechanisms by which GR exerts these effects could provide new insights into therapeutic interventions for PPROM prevention in at risk patients with the use of selective GR agonists and modulators.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

WM performed experiments, data analysis and interpretation, drafting of the manuscript, and approval of the final version of the manuscript. LF contributed to the study concept and design, drafting of the manuscript, and approval of the final version of the manuscript. TA contributed to the study concept and design, performed experiments, data analysis and interpretation, drafting of the manuscript, and approval of the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Interleukin (IL)-6: A Friend or Foe of Pregnancy and Parturition? Evidence From Functional Studies in Fetal Membrane Cells

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Objective: Protection of the fetus within the amniotic sac is primarily attained by remodeling fetal membrane (amniochorion) cells through cyclic epithelial to mesenchymal and mesenchymal to epithelial (EMT and MET) transitions. Endocrine and paracrine factors regulate EMT and MET during pregnancy. At term, increased oxidative stress forces a terminal state of EMT and inflammation, predisposing to membrane weakening and rupture. IL-6 is a constitutively expressed cytokine during gestation, but it is elevated in term and preterm births. Therefore, we tested the hypothesis that IL-6 can determine the fate of amnion membrane cells and that pathologic levels of IL-6 can cause a terminal state of EMT and inflammation, leading to adverse pregnancy outcomes.

Methods: Primary amnion epithelial cells (AECs) were treated with recombinant IL-6 (330, 1,650, 3,330, and 16,000 pg/ml) for 48 h (N = 5). IL-6-induced cell senescence (aging), cell death (apoptosis and necrosis), and cell cycle changes were studied using flow cytometry. Cellular transitions were determined by immunocytochemistry and western blot analysis, while IL-6 signaling (activation of signaling kinases) was measured by immunoassay. Inflammatory marker matrix metalloproteinase (MMP9) and granulocyte-macrophage colony-stimulating factor (GM-CSF) concentrations were measured using a Fluorokine E assay and ELISA, respectively. Amniotic membranes collected on gestational day (D) 12 and D18 from IL-6 knockout (KO) and control C57BL/6 mice (N = 3 each) were used to determine the impact of IL-6 on cell transitions. Fold changes were measured based on the mean of each group.

Results: IL-6 treatment of AECs at physiologic or pathologic doses increased JNK and p38MAPK activation; however, the activation of signals did not cause changes in AEC cell cycle, cellular senescence, apoptosis, necrosis, cellular transitions, or inflammation (MMP9 and GM-CSF) compared to control. EMT markers were higher on D18 compared to D12 regardless of IL-6 status in the mouse amniotic sac.

Conclusion: Physiologic and pathologic concentrations of IL-6 did not cause amnion cell aging, cell death, cellular transitions, or inflammation. IL-6 may function to maintain cellular homeostasis throughout gestation in fetal membrane cells. Although IL-6 is a good biomarker for adverse pregnancies, it is not an indicator of an underlying pathological mechanism in membrane cells.

Keywords: amniotic epithelial cells (AECs), cytokines, EMT, inflammation, fetal membranes

INTRODUCTION

Human fetal membranes (amniochorion) are the innermost lining providing structure as well as immune and mechanical protection to the uterine cavity (Menon, 2016). Membranes start their growth at embryogenesis as two independent layers, i.e., a single layer of amnion epithelium and multilayered chorion trophoblast, which fuse to form the amniochorion, demarcated by a collagen-rich extracellular matrix (ECM) by the early second trimester of pregnancy (Strauss, 2013). Mesenchymal cells are disbursed throughout the ECM and form a key cellular component of the membrane (Strauss, 2013).

During pregnancy, the maintenance of membrane structural integrity is critical to withstand the stretch and stress imposed by the growing volume of the uterine cavity. Highly elastic amnion layer membranes maintain homeostasis through cyclic cellular transitions of amnion epithelial cells (AEC) to amnion mesenchymal cell (AMC) (EMT) and mesenchymal back to epithelial (MET) cells (Richardson et al., 2020). A terminal state of EMT occurs at term where MET is stalled due to oxidative stress (OS)-induced senescence (Richardson et al., 2020) and accumulation of pro-EMT factors like TGFβ in membrane cells and in the amniotic fluid (Richardson et al., 2018). This leads to the accumulation of AMCs in the matrix and promotes localized inflammation and collagenolysis, leading to mechanical weakening (Richardson et al., 2020). This is partly because compared to AECs, AMCs are much more vulnerable to ROS and produce inflammatory mediators (Ko et al., 2012; Denu and Hematti, 2016). Overwhelming membrane inflammation and the propagation of inflammatory mediators to quiescent uterine tissues can transition a quiescent cervix and myometrium into a pro-labor active state (Sheller-Miller et al., 2016; Hadley et al., 2018). Fetal membranes, therefore, play a major role in pregnancy maintenance as well as in promoting parturition. Understanding the factors that maintain membrane cellular, structural, functional, and mechanical integrity is therefore important.

Membranes are rich sources of several biochemical mediators that help to maintain various membrane functions (Menon et al., 2015), although their precise contributions are unclear (Feng et al., 2018). Knowledge of these functional contributions is critical to understanding the physiologic and pathologic contribution of these biochemicals during pregnancy and in term and preterm parturitions. This study is an attempt to understand the functional role of interleukin (IL)-6 in amnion membrane functions. IL-6 is a pleiotropic cytokine that has been documented to perform distinct functions during various stages of pregnancy, including implantation, embryogenesis, pregnancy, and parturition (Prins et al., 2012). Infection and injuries to tissues can increase IL-6 production and generate a host inflammatory response by stimulating acute phase responses (Prins et al., 2012). IL-6 can also control inflammation by minimizing the impact of other inflammatory cytokines such as IL-1β and TNF-α (Prins et al., 2012). IL-6 is produced by human fetal membranes, and its expression increases in both amnion and chorion cells in response to infectious stimuli (Santhanam et al., 1991; Fortunato et al., 1994; Snyers and Content, 1994; Menon et al., 1995; Keelan et al., 1997). IL-6 is also one of the most studied biomarkers in spontaneous preterm birth (PTB) and preterm prelabor rupture of the membrane (pPROM) (Santhanam et al., 1991; Menon et al., 2011). It has been reported that the concentration of IL-6 is increased in the amniotic fluid (Romero et al., 1990), cervical vaginal fluid (Lockwood et al., 1994, 2010), fetal membranes (Santhanam et al., 1991), decidua (Lockwood et al., 2010), myometrium (Rauk et al., 2001), and cervix in PTB and pPROM (Chai et al., 2012), both in cases of microbial invasion of the intraamniotic cavity (MIAC) as well as in the absence of infection (Prins et al., 2012). Recently, many investigators have proposed IL-6 as a predictor of MIAC and intraamniotic inflammation (IAI) in PTB and pPROM (Chaemsaithong et al., 2015, 2016a,b; Kacerovsky et al., 2018). IL-6 is also a member of the senescence-associated secretory phenotype (SASP) family, and IL-6 expression is higher in fetal membranes at term labor compared to term not in labor (Menon et al., 2016). Functionally, the IL-6 knockout model has been shown to lead to delayed term delivery (Robertson et al., 2010), suggesting a pro-parturient function; however, several *in vitro* human cell/tissue-based studies (Mitchell et al., 1991; Kent et al., 1993; Lockwood et al., 2010; Devi et al., 2015) and non-human primate studies (Sadowsky et al., 2006) create ambiguity regarding its exact functional role in promoting parturition either at term or preterm.

IL-6 has also been reported to promote cellular proliferation (Lee et al., 2016) and migration (Jovanovic and Vicovac, 2009), EMT (Lee et al., 2016; Xiao et al., 2017; Browning et al., 2018; Sun et al., 2018), as well as senescence (Kojima et al., 2013). We have earlier reported that human fetal membrane cells, specifically AECs, undergo proliferation, migration, and transitions during pregnancy and aging at term (Richardson and Menon, 2018). However, reported roles of IL-6 are rather vague and this ambiguity regarding its functional role during pregnancy and parturition led us to conduct multiple functional tests in fetal membrane cells. It is likely that IL-6 may play multiple functional roles in regulating membrane homeostasis during gestation or in the promotion of senescence at term. Using an

in vitro model of primary AECs, we tested proliferation and the cell cycle, cellular aging (senescence), cell death (necrosis and apoptosis), cellular transitions, cell signaling, and the generation of inflammation in response to physiologic (term pregnancy, not in labor and labor) and pathologic [spontaneous preterm birth (PTB) and pPROM with MIAC and intraamniotic inflammation (IAI)] concentrations of IL-6 seen in the amniotic fluid.

MATERIALS AND METHODS

IRB Approval

This study protocol was approved by the Institutional Review Board at The University of Texas Medical Branch (UTMB) at Galveston, TX, United States, as an exempt protocol to use discarded placenta after normal term cesarean deliveries (UTMB 11-251). No subject recruitment or consenting was done for this study and no identifiers were collected.

Clinical Samples

Samples we collected from the discarded placentas of term deliveries. Fetal membranes were dissected from the placenta, washed three times in normal saline, and cleansed of blood clots using cotton gauze. Tissue was then processes as described below to isolate fetal membrane cells.

Term Sample Criteria

Placentas from women (18–40 years old) undergoing elective repeat cesarean delivery (between 37 and 41 weeks of gestation) prior to the onset of labor were included in the study. Women with a prior history of preterm labor and delivery, preterm premature rupture of the membranes, preeclampsia, placental abruption, intrauterine growth restriction, and gestational diabetes were excluded. Patients that were group B *Streptococcus* carriers, who were treated for urinary tract infection, sexually transmitted diseases, chronic infections like HIV, hepatitis, and women who smoked cigarettes or reported drug and alcohol abuse were also excluded from this experiment.

Isolation and Culture of Human Amnion Epithelial Cells (AECs)

All reagents and media were warmed to 37°C prior to use. The amnion membrane was manually peeled from normal, term, not in labor cesarean section placentas, then rinsed in saline and transferred to a Petri dish that contained Hanks Balanced Salt Solution (HBSS) (Mediatech Inc., Manassas, VA, United States). The amnion membrane was then cut into 2 cm × 2 cm pieces. They were digested twice in 0.25% trypsin and 0.125% collagenase A (Sigma-Aldrich, St. Louis, MO, United States) in HBSS for 35 min at 37°C. After each digestion, the tissue was filtered through a 70 μm strainer (Fisher Scientific, Waltham, MA, United States) cell and trypsin was inactivated using complete Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 media (DMEM/F12) (Mediatech Inc.) supplemented with 15% fetal bovine serum (FBS) (Sigma-Aldrich), 10% penicillin/streptomycin, 10% amphotericin B (Mediatech Inc.), and 50 μg/mL epidermal growth factor (EGF) (Sigma-Aldrich). The collected filtrate was centrifuged for 10 min at 3,000 *g*. The cell pellet was re-suspended in 5 mL complete DMEM/F12. The cells were then counted using a hemocytometer. Once cells were counted, approximately 3–5 million cells per flask were cultured in T75 flasks containing complete DMEM/F12 media at 37°C, 5% CO_2, and 95% air humidity until they were 80–90% confluent.

AEC Treatment With IL-6

Once the passage zero cells were 80–90% confluent, they were passed into a variety of culture plates depending on the assay and allowed to attach as passage one cells for 12–24 h. After cell were attached by 24 h, cells were treated one time 48-h treatments of IL-6 (330, 1,650, 3,300, and 16,000 pg/mL) or control media (Santhanam et al., 1991). The doses of IL-6 were based on reported concentration ranges during various stages of gestation (330–1,650 pg/mL), term labor (3,300 pg/mL), and preterm labor with and without rupture (16,000 pg/mL) with documented MIAC and inflammation (Musilova et al., 2015). After 48 h cells were collected for a variety of end point assays described below.

Decidua Cell Culture and Validation of Functionality of Recombinant IL-6

Separation of primary decidua cells from the chorio-decidua membrane involved blunt dissection with forceps and a scalpel. The decidua was minced by cross-cutting with scalpel blades. Tissues were processed in a digestion buffer containing 0.125% trypsin (Cat# 85450c, Sigma), 0.2% collagenase (Cat# C0130, Sigma), and 0.02% DNase I (Cat# DN25, Sigma) and incubated at 37°C for 60–90 min. Samples were subsequently neutralized with complete medium (1:1 mixture of Ham's F12/DMEM, supplemented with 5% heat-inactivated FBS, 10 ng/mL EGF, 100 U/ml penicillin G, and 100 mg/mL streptomycin) (Cat# 30-001-CI, Corning). After filtration, the cell solution was centrifuged at 3,000 rpm for 10 min. A cell-separation gradient was prepared using an Optiprep column (Axis-Shield), with steps ranging from 4 to 40% of 4 mL each (4, 6, 8, 10, 20, 30, and 40%). Processed decidual cells were added to the top of the gradient and centrifuged (3,000 × *g*) at room temperature for 35 min. Cell densities of 1.027–1.038 g/mL represented the decidua layer. Harvested cells were washed with DMEM, centrifuged, resuspended in DMEM, and plated at a density of 80,000 (decidua) per well to yield cultures with 95–99% purity. Decidua cells were allowed to attach for 12–24 h and then treated with various concentrations of IL-6 for 12. After 48 h of incubation, the cells were lysed and stained for STAT3 by western blot to confirm IL-6 potency (Devi et al., 2015) (**Supplementary Figure S1**).

Multiplex Assay for Protein Kinases

Medium that was collected from the IL-6 treated cells were used in the Milliplex MAP cell signaling buffer and detection kit for magnetic beads (Millipore, Burlington, MA, United States) (i.e., Phospho JNK, ERK, p38, ATF2, MSK1, c-JUN, STAT1, and HSP27) ($N = 5$). Media and substrates were added to the kit plate

and instructions were followed per kit protocol. Briefly, assay buffer was added to the plate, then removed. Then, magnetic beads, assay buffer, and sample lysates were added to the plate and were incubated for 16–20 h at 4°C on a shaker in the dark. The buffer was then removed and the beads were washed twice using assay buffer. 1X Milliplex MAP detection antibody was added to the plate and incubated on a shaker for 1 h at room temperature (20–25°C) in the dark. The detection antibody was then removed, and 1X streptavidin-PE was added and incubated for 15 min on the plate shaker in the dark. Amplification buffer was then added. The plate was run using a Luminex 200 (LX200-XPON-IVD, Luminex Corporation, Austin, TX, United States) apparatus. Data was then analyzed.

Flow Cytometry Assays

Cell Cycle Analysis

Cells were plated in 12-well plates, with 30,000 cells per well (N = 3). The cells were left to attach for 12–24 h after the cells were plated, then they were treated with IL-6 at different concentrations for 48 h. Cells were collected by adding trypsin, then trypsinization was stopped using complete medium. Cells were then transferred to a conical tube. Cells were centrifuged at 2,000 *g* for 5 min. A cell cycle assay kit (c03551, Beckman Coulter, Brea, CA, United States) was used. Propidium iodine were added to the cells, and they were run on a flow cytometer.

AEC Senescence Flow

Cells were plated in 6-well plates, with 300,000 cells per well (N = 3). The cells were left to attach for 12–24 h after the cells were plated, then they were treated with IL-6 at different concentrations. At the 48-h endpoint, the medium was removed. Cells were treated with diluted bafilomycin (cat# BML-CM110-0100, Enzo Life Sciences, Farmingdale, NY, United States) and the negative control was treated with DMSO. Cells were incubated at 37°C for 1 h. C12FDG (cat#7188 Setareh Biotech, Eugene, OR, United States) was added to the cells, except for the negative control. Cells were collected by adding trypsin, then trypsinization was stopped using complete medium. Cell were then transferred to a conical tube and centrifuged at 3,000 *g* for 10 min. The medium was removed and the pellet was re-suspended in 300 μL of annexin buffer treated with propidium iodine. The cells were run on a flow cytometer using a standard senescence-associated-β-galactosidase template (SA-β-Gal).

AEC Apoptosis and Necrosis

Cells were plated in 12-well plates, with 30,000 cells per well (N = 3). The cells were left to attach for 12–24 h after the cells were plated, then they were treated with IL-6 at different concentrations for 48 h. At the 48-h endpoint, the medium was removed. Cells were collected by adding trypsin, then trypsinization was stopped using complete medium. Cell were then transferred to a conical tube and centrifuged at 2,000 × *g* for 5 min. The medium was removed and the pellets were re-suspended in 100 μL of annexin buffer and treated with propidium iodide and Alexa Fluor 488 annexin-V from the Alexa Fluor 488 Annexin-V/Dead 1 Kit (V13241, Fisher Scientific, Hampton, NH, United States). The cells were run on a flow cytometer; Q2-UR represents necrotic cells and Q2-LR represents apoptotic cells.

Microscopy

Brightfield Microscopy

Brightfield microscopy images were captured using a Nikon Eclipse TS100 microscope (4, 10, and 20×) (Nikon, Melville, NY, United States). Three regions of interest per condition were used to determine the overall cell morphology.

Fluorescence Microscopy

Fluorescent microscopy images were captured using a Keyence All-in-one Fluorescence BZ-X810 microscope (Keyence Corporation of America, Itasca, IL, United States). Three regions of interest per condition were used to determine the ratio of cytokeratin-18 and vimentin.

AEC Immunohistochemistry for EMT Markers

Cells were plated in 8-well glass coverslip, with 50,000 cells per well (N = 5). The cells were left to attach for 12–24 h after the cells were plated, then they were treated with IL-6 at different concentrations. At the 48-h endpoint, the cells were fixed in 4% PFA, then washed with PBS and 0.5% Triton-X. The cells were blocked using 3% BSA, and primary antibodies were added: cytokeratin-18 (CK-18, 1:800, ab668, Abcam, Inc. Cambridge, MA, United States) and vimentin (1:300, ab92547, Abcam Inc. Cambridge, MA, United States). The cells were then washed with PBS and DAPI was added. The images were then captured using fluorescent microscopy at 20×. Uniform laser intensity and brightness/contrast analysis was conducted on vimentin and CK-18 levels to determine the vimentin/CK-18 ratio (Richardson and Menon, 2018; Richardson et al., 2019, 2020).

C57/B6 Samples

The IL-6 deficient mice used in this study were previously reported (Poli et al., 1994). Wild type (Jackson Laboratory, Bar Harbor, ME, United States) and IL-6 deficient C57BL/6 mice were housed under AAALAC-approved (IACUC #19-018) and specific pathogen-free conditions using a 12-h light cycle and *ad libitum* food (normal mouse chow) and water. Mice underwent timed mating as previously described (Bonney et al., 2016). Briefly, female mice were seasoned to male pheromones for 3 days and then mated for 18 h although the mating window is typically 4–6 h around midnight, after which the males were removed. Copulatory plugs are checked the following morning (∼18 h later) to confirm mating. Mice are then monitored and a weight gain of >1.75 g by E10 are confirmed pregnant. At D12 and D18 of gestation, pregnant females were euthanized and intrauterine tissues were removed and snap-frozen in liquid nitrogen until tissue lysis was conducted (N = 3). After opening the uterus, fetal tissues and underlying implantation uterus were removed en bloc. The amniotic sacs were dissected away from the fetal placental unit.

Western Blot Analysis

Decidua and mice tissue were lysed with RIPA lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, and 1.0 mM EDTA pH 8.0, 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktail and phenylmethylsulphonyl fluoride. After centrifugation at 10,000 rpm for 20 min, the supernatant was collected. Protein quantification was performed using the Pierce BCA protein assay kit (Thermo Fisher Scientific). The protein samples (N = 3) were separated using precast gels and transferred to the membrane using an iBlot1 gel transfer device (Thermo Fisher Scientific, Waltham, MA, United States). Membranes were blocked in 5% blotting grade blocker made with 1x Tris buffered saline-Tween 20 (TBS-T) buffer for 1 h. The primary antibody was added and the membrane was left to rock overnight at 4°C. The membrane was incubated with a secondary antibody. For membranes that were stripped, restore western blot stripping buffer was used; none of the membranes in this study were stripped more than three times. The following non-human antibodies were used: vimentin (ab92547, Abcam Inc. Cambridge, MA, United States), actin (Sigma-Aldrich, A5441), and STAT3 (SC8019, Santa Cruz, Waltham, MA, United States).

Statistical Analysis

Statistical analysis for normally distributed data, as defined by Prisms normality test, was performed using an ANOVA with Tukey's multiple comparisons test and one-tailed unpaired t-tests. Statistical values were calculated using GraphPad Prism; P-values less than 0.05 were considered significant. Additionally, fold changes were calculated based on data means. Data are represented as mean ± SEM in bar graphs. All data were analyzed using GraphPad Prism 6.

RESULTS

Term Labor IL-6 Concentrations Activate JNK and p38MAPK but Do Not Induce Pro-labor Inflammatory Markers in AECs

To test that the recombinant IL-6 used in our experiments was functionally active, primary decidua cells were treated with a range of IL-6 concentrations and the activation of the downstream transcription factor STAT3 was determined as previously shown by Devi et al. (2015). As expected, IL-6 induced STAT3 expression in these cells, showing their functional viability (**Supplementary Figures S1A,B**).

In order to evaluate the ability of IL-6s to activate various signaling pathways in AECs, a multiplex kinase panel was conducted. Term labor (3,300 pg/mL) IL-6 levels showed increasing trends of upstream kinases JNK and p38MAPK activation compared to control AECs (**Figures 1A,B**), whereas lower doses had no effect on any of the kinases. Additionally, the downstream kinases MSK1, ATF, and c-Jun showed similar activation trends (**Figures 1A,B**). However, activation of these kinases did not induce some of their downstream targets such as pro-labor inflammatory mediators MMP9 and GM-CSF (**Figures 1C,D**) compared to controls, suggesting the high levels of IL-6 do not functionally contribute to the inflammatory onslaught required to induce labor in AECs.

Due to the inability of IL-6 to activate pro-labor kinase pathways leading to inflammation, further experiments were conducted to determine the role of IL-6 in cellular processes in AECs.

Gestational Concentrations of IL-6 Do Not Induce Changes in the Cell Cycle, Cellular Aging, Cell Death, or Cellular Transition in AECs

IL-6 treatments mimicking early (300 pg/mL), mid (1,650 pg/mL), and term (3,300 pg/mL) gestation were used to determine if IL-6 could induce cell cycle-related changes in AEC, as reported in other systems (Santhanam et al., 1991). Cell cycle analysis by flow cytometry determined that physiologic doses of IL-6 did not induce any changes in G0, G1, S phase, or G2 and compared to control cells (**Supplementary Figure S2**).

Regardless of concentration, IL-6 did not induce senescence, as noted by no change in senescence-associated-β-galactosidase (SA-β-Gal) positive cells (**Figure 2A**). Furthermore, IL-6 did not induce the two main types of cell deaths, i.e., necrosis and apoptosis. As shown in **Figure 2B**, flow cytometry data showed no change in IL-6-induced necrosis as determined by annexin-V + propidium iodide (PI) negative cells or apoptosis as determined by a lack of annexin-V and P-p53.

Given that our data did not show that IL-6 induced changes to cell cycle, caused cellular aging or cell death, attention was turned to determine if IL-6 induced cellular transitions (EMT or MET) in AEC. AECs were stained with both cytokeratin-18 (CK-18; red; epithelial marker) and vimentin (green; mesenchymal marker) to determine the transition status based on the vimentin/CK-18 ratio after IL-6 treatment (**Figure 2C**). A high vimentin/CK-18 ratio indicates a mesenchymal phenotype, while a low ratio indicates an epithelial phenotype (Richardson and Menon, 2018; Richardson et al., 2019, 2020). Irrespective of concentration, IL-6 caused a slight change in morphology (see inset images) and the vimentin/CK-18 ratio that kept AECs in a "metastate" (Richardson and Menon, 2018) or native state where they co-expressed both epithelial and mesenchymal markers (**Figure 2C**).

Based on these data, pathologic doses of IL-6 (16,000 pg/mL) reported in microbial invasion and intraamniotic inflammation were investigated to see if this dose of IL-6 activated pro-labor pathways leading to inflammation.

IL-6 Concentration Mimicking Infectious and Inflammatory pPROM Activates JNK and p38MAPK but Does Not Induce Pro-labor Inflammatory Markers in AECs

A kinase immunoassay was performed to assess IL-6 concentrations within amniotic fluid from infectious pPROM

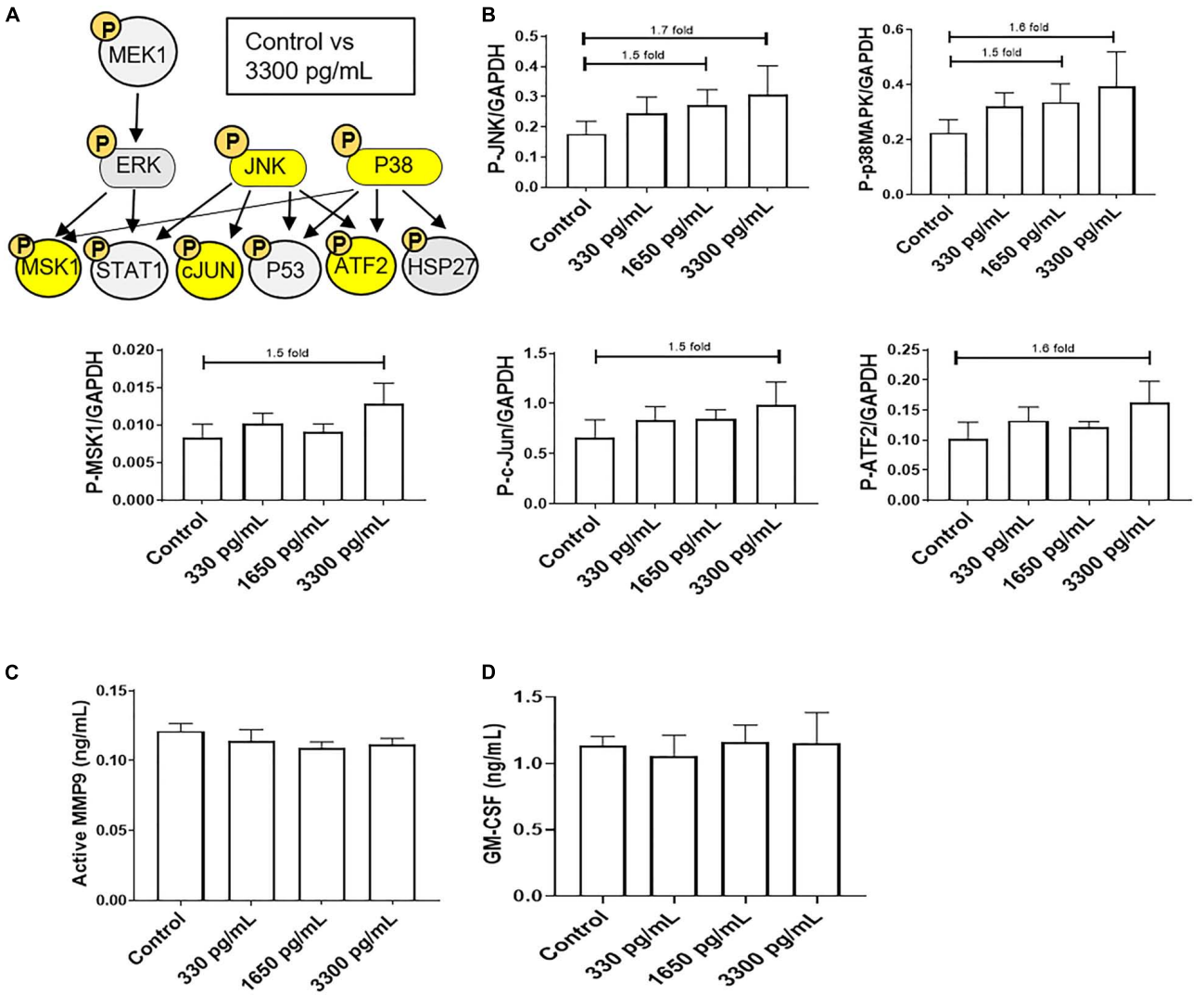

FIGURE 1 | IL-6 dose reported in term labor amniotic fluid activates JNK and p38MAPK, leading to downstream kinase activation in AECs. **(A)** Schematic of various phosphorylated (P) kinases tested using a multiplex immunoassay. Highlighted (yellow) P-proteins were upregulated, though not significantly, after IL-6 treatment mimicking term labor (3,300 pg/mL) compared to control-treated AECs. **(B)** IL-6 treatment (3,300 pg/mL) of AECs showed increasing trends of upstream kinases activation, JNK (1.7-fold) and p38MAPK (1.6-fold), which contributed to the downstream activation of MSK1 (1.5-fold), c-JUN (1.5-fold), and ATF2 (1.5-fold) compared to control samples. *N* = 5; mean ± SEM medium florescent intensity. **(C,D)** IL-6 did not increase the inflammatory profile of AECs, as seen by no changes in MMP9 activation or GM-CSF production when compared to control or term labor IL-6 treatment (3,300 pg/mL). *N* = 5; mean ± SEM.

samples (16,000 pg/mL) to determine if pro-labor pathways were activated (**Figure 3**). Pathologic and physiologic (3,300 pg/mL) IL-6 levels both showed increasing trends of upstream kinases JNK and p38MAPK activation, and the downstream kinase ATF2, compared to control AECs (**Figures 3A,B**). However, pathologic doses of IL-6 also showed increasing trends of downstream kinases STAT1 and HSP27 upregulation compared to control AECs (**Figures 3A,B**). Regardless of IL-6 treatment, AECs did not activate or increase expression of the pro-inflammatory mediators MMP9 and GM-CSF (**Figures 3C,D**).

To further confirm the contribution of the pPROM IL-6 level contributing to a labor phenotype, we tested the effect of IL-6 on AEC cell cycle, cell death, cell aging, and cellular transition.

IL-6 Concentration Mimicking Infectious and Inflammatory pPROM Does Not Induce Changes in Cell Cycle, Cellular Aging, Cell Death, or Cellular Transition in AECs

Treatment with 16,000 pg/mL IL-6, a concentration often seen in infection and inflammation associated pPROM, was used to determine if such a high dose IL-6 could induce AEC cellular pathologies compared to the term labor concentration (3,300 pg/ml). Similar to our observations reported above, cell cycle analysis determined that pathologic doses of IL-6 did not induce any changes

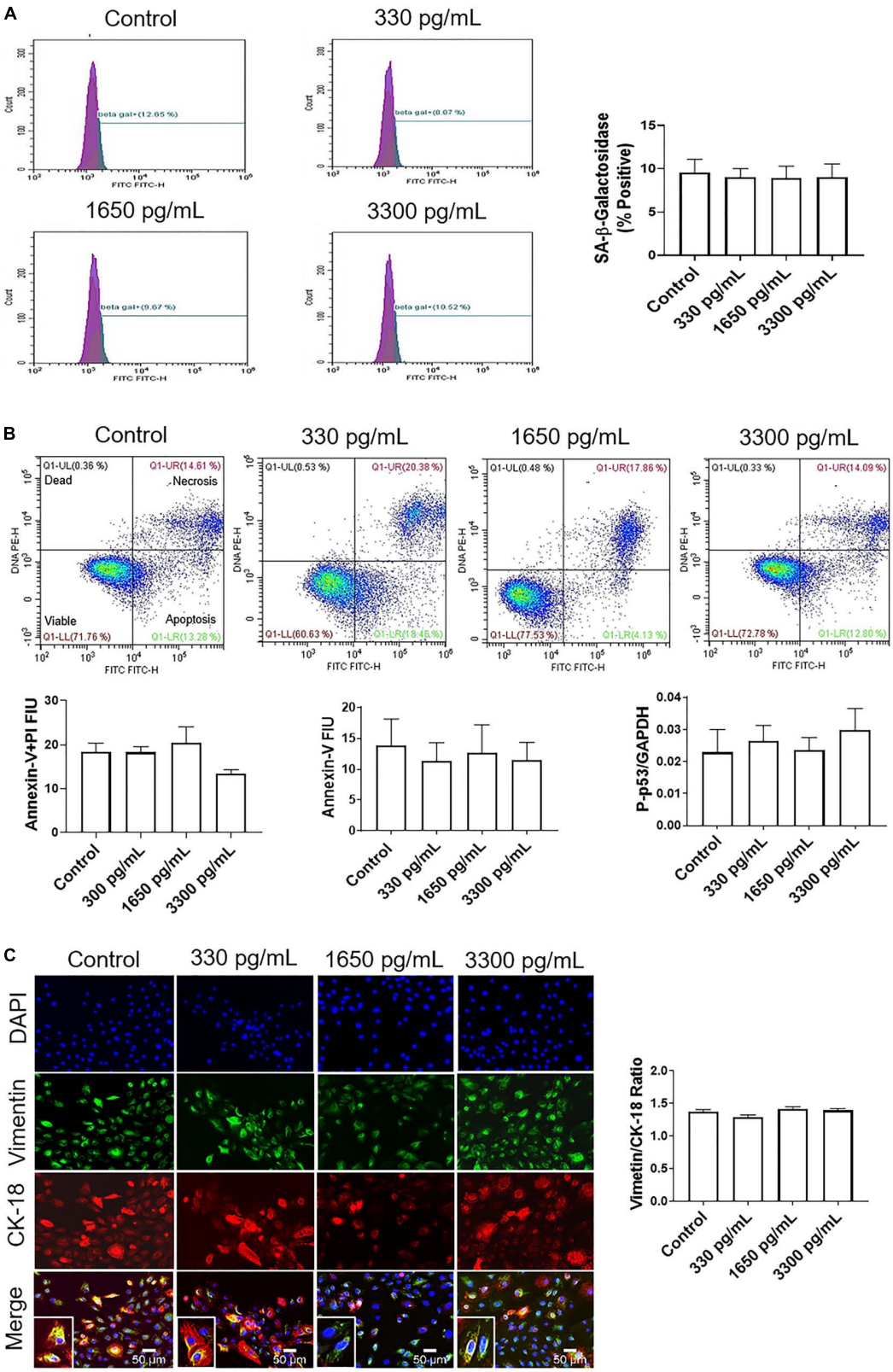

FIGURE 2 | IL-6 at term labor does not induce cellular aging, cell death, or cellular transitions in AECs. **(A)** Flow cytometry analysis of IL-6 induced senescence in AECs. Physiologic concentrations of IL-6 did not increase the population of senescence-associated-β-galactosidase (SA-β-Gal) cells compared to controls. *N* = 5;

(Continued)

FIGURE 2 | Continued
mean ± SEM. **(B)** Flow cytometry analysis of necrotic and apoptotic cells. Physiologic (330, 1,650, and 3,300 pg/mL) concentrations of IL-6 did not induce necrosis, as indicated by a lack of changes in annexin-V + propidium iodide (PI) positive cells (top right box), nor apoptosis as indicated by no changes in Annexin-V (bottom right box) expression between IL-6 treated and untreated AECs. A protein kinase panel further confirmed the lack of apoptosis by showing that phospho-p53, a pro-apoptotic marker, did not change after treatment with IL-6. Fluorescence intensity units (FIU) $N = 3$; mean ± SEM. **(C)** Immunocytochemistry of intermediate filaments in AEC to assess cell transition. AECs were stained with both cytokeratin-18 (CK-18; epithelial marker) and vimentin (mesenchymal marker) to determine transition status after physiologic (330, 1,650, and 3,300 pg/mL) IL-6 treatments. Uniform intensity analysis was conducted on vimentin and CK-18 levels to derive the vimentin/CK-18 ratio. A high vimentin/CK-18 ratio indicates a mesenchymal phenotype, while a low ratio indicates an epithelial phenotype. IL-6 treatment did not change the vimentin/CK-18 ratio compared to control cells. Fluorescent images were captured at 20×. Red: cytokeratin-18 (CK-18), green: vimentin, blue: DAPI. $N = 5$; mean ± SEM. Scale bar 50 μm.

in G0, G1, S phase, or G2 compared to control cells (**Supplementary Figure S2**).

Additionally, flow cytometry indicated a lack of senescence (lack of change in SA-β-Gal activity), necrosis (lack of annexin-V + PI cells), or apoptosis (low annexin-V) (**Figures 4A,B**) compared to either 3,300 pg/ml or control. Lack of apoptosis was further validated by a protein kinase panel showing no change in phosphorylated-P53, a pro-apoptotic protein, regardless of the dose of treatment (**Figure 4A**). Given that our data show that IL-6 did not induce changes in the cell cycle, cellular aging, or cell death, we next determined if IL-6 induced cellular transitions (EMT or MET) in AEC.

Treatment with pathologic levels of IL-6 did not significantly change the vimentin/CK-18 ratio compared to controls and maintained the AEC metastate (**Figure 4C**). When compared to the term labor concentration, 16,000 pg/mL of IL-6 did not increase mesenchymal marker vimentin and maintained the cells in a cuboidal morphology (see inset), suggesting that IL-6 could play a role in AEC homeostasis (**Figure 4C**) rather than EMT induced cellular pathologies as we hypothesized.

To determine if IL-6 induced EMT at term or preterm *in vivo*, we conducted experiments using an IL-6 knockout (KO) mouse model.

IL-6 KO in C57B/6 Mice Does Not Affect EMT at Term

Recently, it was reported that human fetal membranes and mouse amniotic sacs both undergo senescence (Bonney et al., 2016; Polettini et al., 2018) as well as EMT at term labor (Richardson et al., 2020). Since the contribution of IL-6 is unclear for these two events, we utilized an established IL-6 knockout (KO) mouse (Gomez-Lopez et al., 2016) model to determine if IL-6 was needed to induce EMT at term in C57B/6 mice. Western blot analysis and densitometry of the mesenchymal marker vimentin were conducted on amniotic sacs of control C57B/6 mice and KO IL-6 mice on day 12 (D12) (mid-gestation) and day 18 (D18) (term) of gestation (**Figure 5A**). Amniotic sacs from control and IL-6 KO C57B/6 mice showed an 11- and 7-fold increase in vimentin on D18 compared to D12 (**Figure 5A**), respectively, as previously document in CD1 models (Richardson et al., 2020), suggesting the development of a terminal state of EMT as seen in human membranes at term labor. The absence of any significant changes in EMT marker expression in IL-6 KO mice suggests that IL-6 does not control the induction of EMT at term.

DISCUSSION

IL-6, often considered a pro-inflammatory cytokine, is one of the most studied cytokines in human parturition as well as in adverse pregnancy conditions (Menon et al., 2011). IL-6 shows constitutive expression in the uterine tissues during gestation and is elevated during parturition (Romero et al., 1990; Santhanam et al., 1991; Keelan et al., 1997; Lee et al., 2011; Devi et al., 2015); however, its exact function in different uterine compartments is still unclear. Irrespective of etiologies, IL-6 concentration is increased in various biological compartments in PTB and pPROM. IL-6 is also reported to increase prostaglandin productions from AECs and often referred in the literature as a "causal factor" for various adverse outcomes, specifically infection and inflammation associated PTB and pPROM (Romero et al., 1990; Santhanam et al., 1991). As our laboratory is interested in exploring the biology and function of fetal membrane cells as detailed in the introduction, we tested the fate of AECs, a key component of fetal membranes, in response to both physiologic and pathologic doses of IL-6 as a measure of its function. Using an *in vitro* AEC and *in vivo* mouse models, we report the following principal findings; (1) physiologic or pathologic concentrations of IL-6 increased cell signaling kinases (JNK, p38MAPK, ATF2, HSP27, c-JUN, MEK1, and STAT1) involved in various cellular functions, (2) activation of various signaling molecules by IL-6 did not result in the activation of inflammatory mediators, (3) IL-6 does not induce changes to the cell cycle, cellular aging (senescence), or cell death (necrosis and apoptosis), (4) IL-6 did not cause EMT in AECs and cells remained more epithelioid even at pathologic doses, suggesting that IL-6 is definitely not a pro-EMT cytokine in AECs, and (5) EMT was evident in the amniotic sac of mice on D18 compared to D12, confirming our prior reports (Richardson et al., 2020); however, in this study, IL-6 KO mice showed no difference in this pattern, suggesting that IL-6 does not impact the development of EMT at term.

Pregnancy and parturition involve a complex interplay between various endocrine, paracrine, and autocrine systems to maintain homeostasis. Disruptions to any of these processes will lead to adverse pregnancy outcomes such as PTB and pPROM, which are associated with ∼10.5% of all pregnancies around the globe (Blencowe et al., 2012, 2013; Lawn et al., 2013). Endocrine mediators such as progesterone are involved in pregnancy maintenance and parturition (Kota et al., 2013). Similarly, cytokines are a large class of secretory proteins implicated as endocrine, paracrine, and autocrine signaling mediators. Several

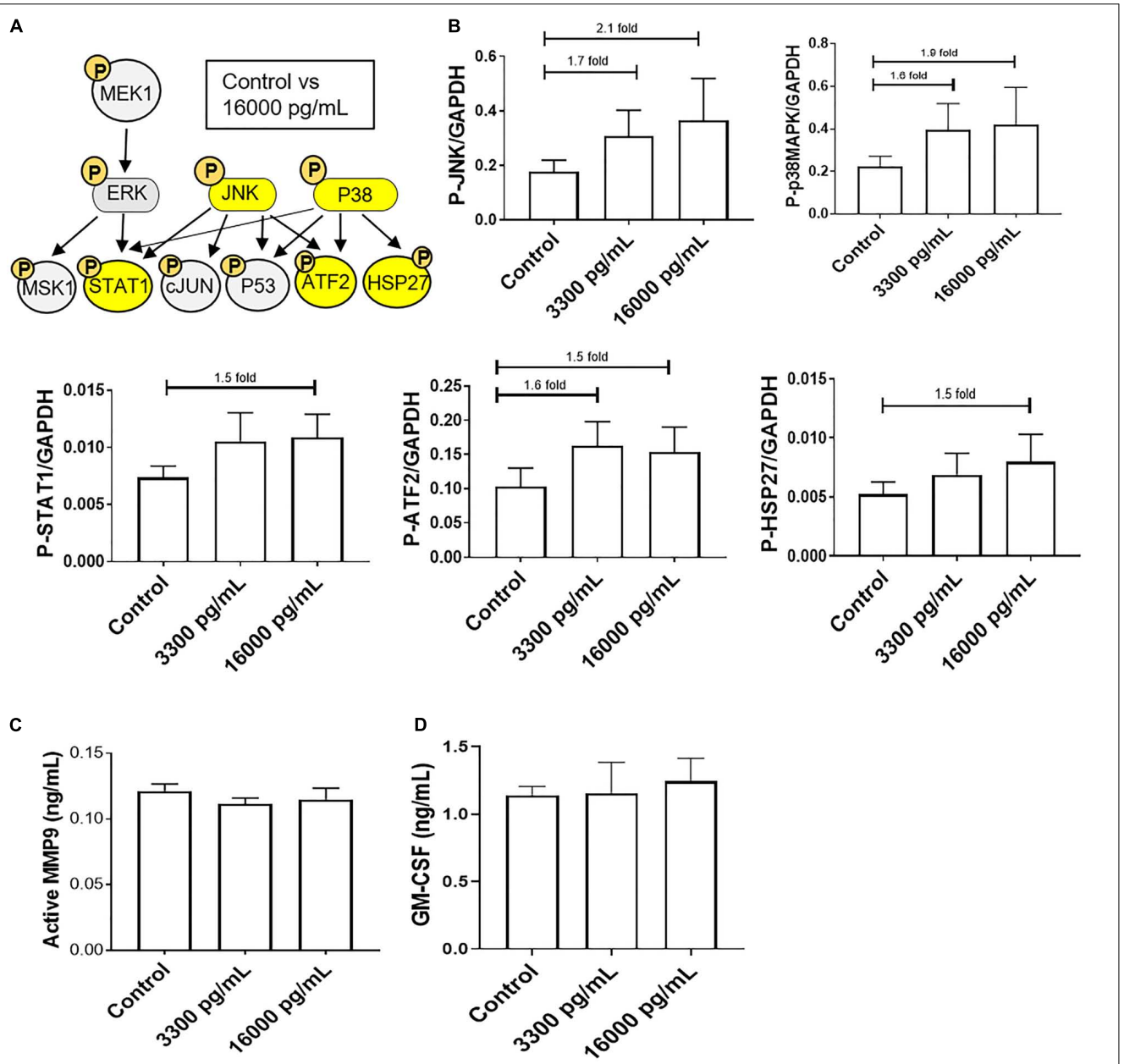

FIGURE 3 | IL-6 concentration reported in the amniotic fluid of infection associated pPROM activates JNK and p38MAPK leading to differential downstream kinase activation in AECs. **(A)** Schematic of various phosphorylated (P) kinases tested using a multiplex immunoassay. Highlighted (yellow) P-proteins were upregulated, though not significantly, after a high dose of IL-6 treatment (16,000 pg/mL) compared to control-treated AECs. **(B)** IL-6 treatment (16,000 pg/mL) of AECs showed increasing trends of upstream kinases activation, JNK (2.1-fold) and p38MAPK (1.9-fold), which contributed to the downstream activation of STAT1 (1.5-fold), ATF2 (1.5-fold), and HSP27 (1.5-fold) compared to control samples. *N* = 5; mean ± SEM medium florescent intensity. **(C,D)** A pathologic dose of IL-6 did not increase the inflammatory profile of AECs, as seen by no changes in MMP9 activation or GM-CSF production when compared to control or term labor IL-6 treatment (3,300 pg/mL). *N* = 5; mean ± SEM.

cytokines have been functionally linked to various biological processes that maintain pregnancy and promote parturition. The most discussed cytokine is IL-6, as it is often reported as a biomarker and implicated in pathological pathways leading to adverse pregnancy outcomes (Romero et al., 1990; Santhanam et al., 1991; Chaemsaithong et al., 2016a). Lack of disruptive roles in cell cycle and cell death, it is likely that IL-6 is involved in pregnancy maintenance although the precise mechanistic functions, if any, are yet to be determined. Additionally, IL-6 could play a role with other cytokines, endocrine, paracrine, and autocrine signaling mediators to induce responses in AECs that were not evaluated in this study.

We were specifically interested in the role of IL-6 in cellular transitions, as this is one of the key mechanisms that

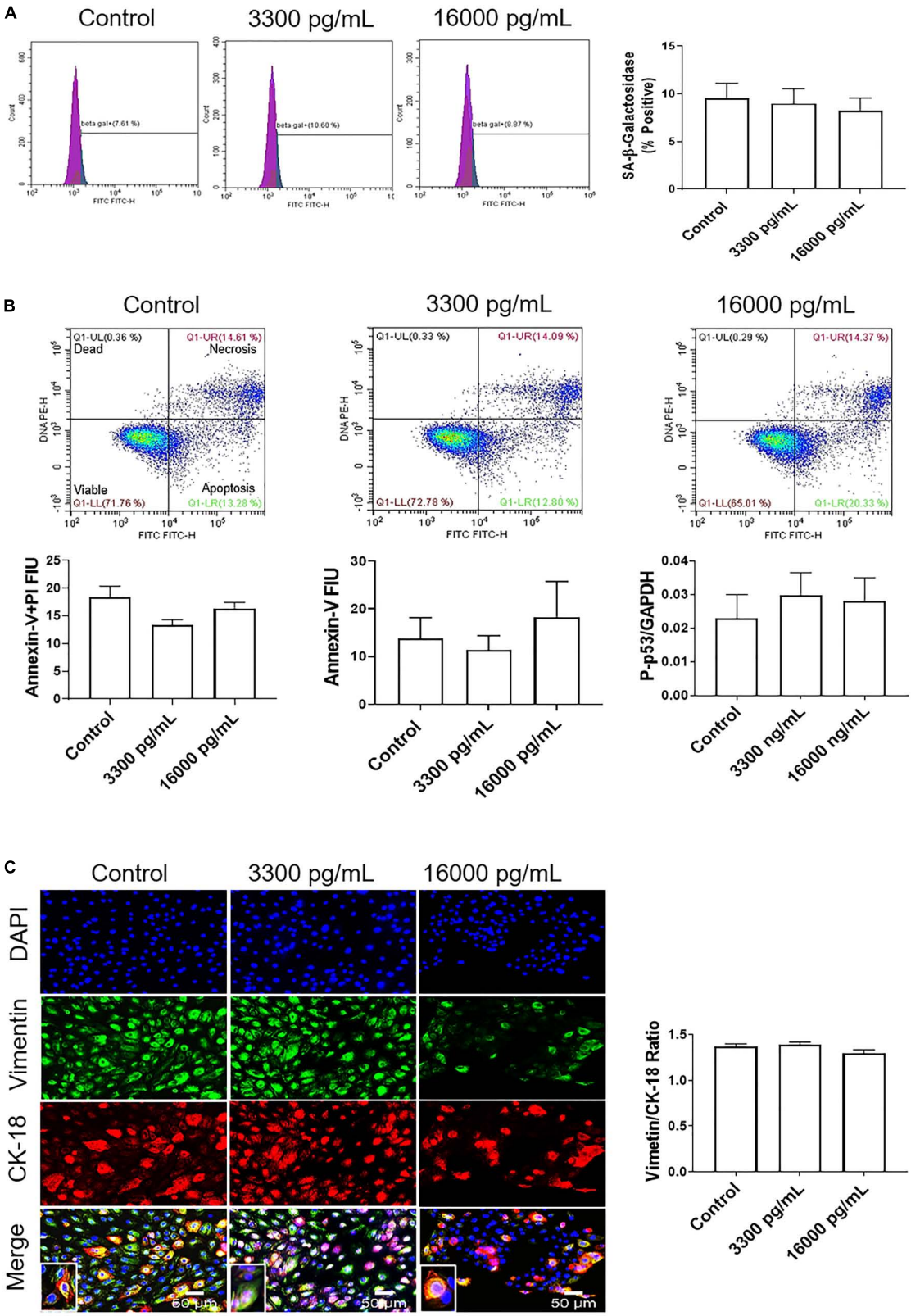

FIGURE 4 | IL-6 concentration reported in the amniotic fluid of infection associated pPROM does not induce cellular aging, cell death, or cellular transitions in AECs. **(A)** Flow cytometry analysis of IL-6 induced senescence in AECs. Physiologic and pathologic concentrations of IL-6 did not increase the population of

(Continued)

FIGURE 4 | Continued
senescence-associated-β-galactosidase (SA-β-Gal) positive cells compared to controls. *N* = 5; mean ± SEM. **(B)** Flow cytometry analysis of necrosis and apoptosis of cells. Physiologic (3,300 pg/mL) and pathologic (16,000 pg/mL) concentrations of IL-6 did not induce necrosis, as indicated by a lack of changes in annexin-V + propidium iodide (PI) positive cells (top right box), nor apoptosis, as indicated by no changes in Annexin-V (bottom right box) expression between IL-6 treated and untreated cells. A protein kinase panel further confirmed a lack of apoptosis by showing that phospho-p53, a pro-apoptotic marker, did not change after treatment with IL-6. Fluorescence intensity units (FIU). *N* = 5; mean ± SEM. **(C)** Immunocytochemistry of intermediate filaments in AEC to assess cell transition. AECs were stained with both cytokeratin-18 (CK-18; epithelial marker) and vimentin (mesenchymal marker) to determine transition after physiologic and pathologic IL-6 treatment. Uniform intensity analysis was conducted on vimentin and CK-18 levels to derive the vimentin/CK-18 ratio. A high vimentin/CK-18 ratio indicates a mesenchymal phenotype, while a low ratio indicates an epithelial phenotype. IL-6 treatment did not change the vimentin/CK-18 ratio compared to control cells. Fluorescent images were captured at 20×. Red: cytokeratin-18 (CK-18), green: vimentin, blue: DAPI. *N* = 5; mean ± SEM. Scale bar 50 μm.

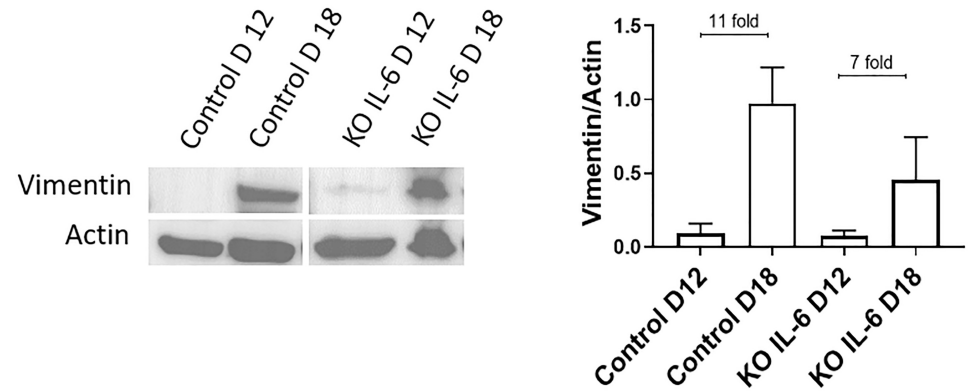

FIGURE 5 | Normal and IL-6 KO mice undergo EMT associated changes at term. Western blot analysis and densitometry of mesenchymal marker vimentin in the amniotic sac of C57B/6 mice and KO IL-6 mice on day 12 (mid-gestation) and day 18 (term) of gestation. Amniotic sacs from control C57B/6 mice showed an increase in vimentin on day 18 compared to day 12 (11-fold), as previously document in CD1 models. The absence of IL-6 did not inhibit the increase in vimentin on day 18 compared to day 12 (7-fold). *N* = 3 for each category; mean ± SEM.

recycles cells during gestation. Recent findings support the concept that AECs are constantly shed from the surface of the membrane and do not have a uniform surface (Richardson et al., 2017b,c). As the membranes grow, AECs undergo replicative senescence and become inflamed (Menon et al., 2013; Bredeson et al., 2014; Dixon et al., 2018; Hadley et al., 2018). These cells are often seen in the amniotic fluid and have been used in the past for cytogenetic analysis. Local inflammation in the membrane and increases in cytokines such as TGFβ (Richardson et al., 2018) can force them to transition into AMCs (Richardson et al., 2019, 2020). AMCs are highly mobile and find their way into the ECM through the recently reported microfractures in the membranes (Richardson et al., 2017c). As a part of the membrane remodeling process, AMCs are recycled and transitioned back to AECs (Richardson et al., 2019). This is mainly driven by progesterone through its membrane receptor 2 (PGRMC2) and mediated by c-MYC activation (Richardson et al., 2020). Cellular recycling, along with localized inflammation that rebuilds the ECM, helps to maintain membrane integrity. Reduced AMC recycling and their subsequent accumulation in the matrix occur at term prior to labor due to a "functional P4 withdrawal" (Richardson et al., 2020). This process is specific to the membranes and is due to PGRMC2 downregulation and not due to a change in phosphorylation status, as has been reported in the myometrium. OS at term and infection/inflammation markers can also force the downregulation of PGRMC2-mediated P4 withdrawal, creating a terminal state of EMT (Richardson et al., 2020). Since IL-6 is available at a baseline level in the amnion membranes under normal conditions (Keelan et al., 1997) and IL-6 increases following stimulation of any kind, we were specifically interested in its contribution to determining amnion membrane cell fate. Our data suggest that while IL-6 may still be a reliable marker for infection/inflammation in PTB and pPROM, it does not functionally contribute to the disruption of cellular homeostasis in the membranes.

Based on our data, both physiologic and pathologic doses of IL-6 showed a tendency to maintain the epithelial state, suggesting that endogenous IL-6 may play a role in maintaining the metastate of the cell rather than promoting any type of transition. We confirmed this hypothesis using an *in vivo* IL-6 KO mouse model. Analysis of amniotic sacs from IL-6 KO mice further confirmed our findings as the loss of IL-6 did not affect the expression of transition markers. These data suggest that, during a healthy pregnancy, baseline levels of IL-6 are an indicator or by-product of an ongoing inflammatory process; IL-6 may perhaps support pregnancy maintenance but not necessarily directly contribute to any of these functions. IL-6 activated various signaling molecules such as JNK, p38MAPK, MSK1, STAT1, HSP27, c-JUN, and ATF2, confirming its activity in AECs. Downstream targets of these signaling molecules include pro-inflammatory cytokines, prostaglandins, and matrix remodeling enzymes (Pirianov et al., 2015; Menon and Papaconstantinou, 2016; Cutler et al., 2017). In our study, activation of these

signaling molecules by IL-6 did not result in a pro-inflammatory environment, suggesting that IL-6 alone, at doses that are often associated with normal and abnormal pregnancies, is not capable of inducing a pro-parturition status.

As mentioned above, IL-6 is a well-reported biomarker of inflammation and intraamniotic infection (Combs et al., 2015). Point of care (POC) tests using specific concentrations of IL-6 have been developed, and some are in clinical use (Chaemsaithong et al., 2015, 2016a,b; Kacerovsky et al., 2018). POC tests primarily use amniotic fluid to diagnose the status of MIAC and IAI and assist clinicians to provide better management in cases with preterm labor and or pPROM (Chaemsaithong et al., 2015, 2016a,b; Kacerovsky et al., 2018). These studies showed ethnic and other regional variations in IL-6 concentrations (Graham et al., 2018), and standard of care has been developed using population-specific values of IL-6 seen in amniotic fluids. Based on our data, the value of IL-6 as a biomarker does not change, as tissues respond to various stressors with substantial production of IL-6 indicative of an underlying pathology. However, this increase may not be indicative of any specific risk exposure or a pathobiological problem with either the mother or the fetus. Along with IL-6, additional biomarkers indicative of specific risks may be needed to provide risk-targeted intervention. In summary, IL-6 is not an indicator of changes related to any specific pathologic or physiologic function, but it is a good indicator of an overall disturbance during pregnancy that can alert clinicians to design management strategies. Sadowsky et al. (2006) has shown that an infusion of IL-6 into pregnant non-human primates does not cause preterm labor, whereas IL-1β and TNFα are better inducers of labor in this model.

We have earlier shown in AECs that IL-6 is functionally a very complex cytokine and performs its functions either through classical or *trans*-signaling mechanisms, which determine its biological activities (Noda-Nicolau et al., 2018). Classical signaling, through the membrane-bound IL-6 receptor (mIL-6R), produces an anti-inflammatory effect; conversely, *trans*-signaling through the soluble IL-6 receptor (sIL-6R) generates a pro-inflammatory effect (Noda-Nicolau et al., 2018). In both cases, the binding between IL-6 and mIL-6R or sIL-6R is followed by homodimerization of the β-subunit of glycoprotein 130 (gp130) (Lee et al., 2011; Rose-John, 2012), the signal transduction subunit. However, soluble gp130 (sgp130) functions as a natural antagonist of IL6/sIL6R *trans*-signaling, neutralizing IL-6 bioactivity by blocking IL-6/sIL-6R complex binding to gp130 and suppressing inflammation (Noda-Nicolau et al., 2018). We have tested the role of IL-6s in polymicrobial infection (genital mycoplasmas and *Gardnerella vaginalis*) of the fetal membranes (Noda-Nicolau et al., 2016). Our study reported that genital mycoplasmas alone, or in combination, inhibited IL-6 *trans*-signaling with increased sgp130 production. *G. vaginalis* activated the classical IL-6 signaling pathway (Noda-Nicolau et al., 2018). Polybacterial treatment resulted in a balanced response with neither pathway being favored. This study did not examine changes associated with IL-6 receptors that may play a role in mediating IL-6 functions.

We modeled our study using AECs, and the data and discussion are restricted to the function of IL-6 on these cells. Fetal membrane matrix also contains innate immune cells (∼2%) and our study did not test IL-6 and its receptors in these cells. AECs are constantly exposed to rapid changes in the neighboring environment during pregnancy as they are the innermost lining of the amniotic cavity and are constantly bathed in amniotic fluid (Richardson et al., 2017a; Menon et al., 2018). These changes in the environment include, but are not limited to, the redox state, endocrine factors, cytokines, chemokines and growth factors, feto-maternal immune cells, and extracellular vesicles carrying various cargoes that can also induce functional changes (Menon, 2019). Besides these biochemicals, mechanical stretching (due to growing fetal and amniotic fluid volume) and fetal movements can also impact amnion membrane cells (Joyce et al., 2016). Therefore, the impact on these cells provides valuable information on their behavior when in an environment with normal as well as high dose of IL-6. This is also a limitation of this study, as IL-6 may have other functional roles in other cell types in the uterine cavity, specifically in AMCs and the choriodecidua layer. AMCs residing in the stroma, are very reactive to pro-inflammatory stimulants; however, in the absence of rupture, any pro-inflammatory function of IL-6 maybe muted by AEC prior to that reaching AMC or other layers of the membranes. Additionally, IL-6 is hypothesized to play an important role during infection induce chorioamnionitis by activating immune cells within the choriodecidua layer. However, none of these are shown in any reliable and reproducible *in vitro* or animal models and the functional contributions of IL-6 in other reproductive tissues still remain as a hypothesis.

The above discussion is based on our data in AECs and not necessarily reflect its function in other cells of the uterine cavity. We conclude that the functional contributions of IL-6 are rather muted in AECs during reproduction. In association with other factors (e.g., infiltrating innate immune cells during labor), IL-6 may still exert some functions, but to have its own contributions IL-6 should align with its membrane and soluble receptors. This complexity likely minimizes the impact of this cytokine on AECs or it may be performing a very specific function that is not yet clear. An increase in IL-6 production by fetal membranes and other feto-maternal tissues in response to infection is likely a non-specific innate response and not an indication of a functional mediator of any labor-inducing pathways. Regardless of its function or lack thereof, IL-6 can still be a non-specific indicator and a good biomarker in various pregnancy-associated conditions.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

This study protocol was approved by the Institutional Review Board at The University of Texas Medical Branch (UTMB) at Galveston, TX, United States, as an exempt protocol to use

discarded placenta after normal term cesarean deliveries (UTMB 11-251). No subject recruitment or consenting was done for this study and no identifiers were collected. The animal studies involving mice [Wild type (Jackson laboratories, Bar Harbor, ME, United States) and IL-6 deficient C57BL/6 mice] were reviewed and approved by the University of Vermont's Institutional Animal Care and Use Committee [AAALAC-approved (IACUC #19-018)].

AUTHOR CONTRIBUTIONS

CO and LR conducted experiments, performed data analysis, and drafted the manuscript. TK helped run western blots. GS helped with placental specimens. EB contributed IL-6 KO mouse tissues. RM conceived the project, designed the experiments, provided funding and helped with data analysis and interpretation, and prepared the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00891/full#supplementary-material

FIGURE S1 | IL-6 induced dose-dependent STAT3 activation in primary decidua cells validating its functionality. **(A)** Decidual cells were treated with IL-6, with doses as reported in the literature that can cause STAT3 activation, to validate the recombinant and commercially available IL-6 used for the treatments in our study. Western blot analysis showed that IL-6 treatment increased the activation of the downstream transcription factor STAT3 at pathologic levels compared to control (2.7-fold). *N* = 3; mean ± SEM.

FIGURE S2 | IL-6 does not induce cell cycle changes in AECs. Cell cycle analysis was performed by measuring the DNA content and used to distinguish between different phases of the cell cycle after a physiologic or pathologic dose of IL-6. Fluorescence intensity that directly correlated with the amount of DNA contained in a cell was measured by flow cytometry. Concurrent parameter measurements made it possible to discriminate between S (red), G2 (blue), and mitotic cells (SubG0 in purple and G0 in pink). **(A)** Physiologic doses of IL-6 mimicking early gestation (330 pg/mL), mid-gestation (1,650 pg/mL), and term labor (3,300 pg/mL) do not affect normal cell cycle progression at sub G0, G0, S phase, or G2. Fluorescence intensity units (FIU). *N* = 3; mean ± SEM. **(B)** Pathologic doses of IL-6 seen in the amniotic fluid of infectious pPROM do not affect normal cell cycle progression at sub G0, G0, S phase, or G2 when compared to control or term labor treatments. Fluorescence intensity units (FIU). *N* = 3; mean ± SEM.

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REVIEW
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Telomere-Related Disorders in Fetal Membranes Associated With Birth and Adverse Pregnancy Outcomes

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Telomere disorders have been associated with aging-related diseases, including diabetes, vascular, and neurodegenerative diseases. The main consequence of altered telomere is the induction of the state of irreversible cell cycle arrest. Though several mechanisms responsible for the activation of senescence have been identified, it is still unclear how a cell is indeed induced to become irreversibly arrested. Most tissues in the body will experience senescence throughout its lifespan, but intrinsic and extrinsic stressors, such as chemicals, pollution, oxidative stress (OS), and inflammation accelerate the process. Pregnancy is a state of OS, as the higher metabolic demand of the growing fetus results in increased reactive oxygen species production. As a temporary organ in the mother, senescence in fetal membranes and placenta is expected and linked to term parturition (>37 weeks of gestation). However, a persistent, overwhelming, or premature OS affects placental antioxidant capacity, with consequent accumulation of OS causing damage to lipids, proteins, and DNA in the placental tissues. Therefore, senescence and its main inducer, telomere length (TL) reduction, have been associated with pregnancy complications, including stillbirth, preeclampsia, intrauterine growth restriction, and prematurity. Fetal membranes have a notable role in preterm births, which continue to be a major health issue associated with increased risk of neo and perinatal adverse outcomes and/or predisposition to disease in later life; however, the ability to mediate a delay in parturition during such cases is limited, because the pathophysiology of preterm births and physiological mechanisms of term births are not yet fully elucidated. Here, we review the current knowledge regarding the regulation of telomere-related senescence mechanisms in fetal membranes, highlighting the role of inflammation, methylation, and telomerase activity. Moreover, we present the evidences of TL reduction and senescence in gestational tissues by the time of term parturition. In conclusion, we verified that telomere regulation in fetal membranes requires a more complete understanding, in order to support the development of successful effective interventions of the molecular mechanisms that triggers parturition, including telomere signals, which may vary throughout placental tissues.

Keywords: telomere shortening, oxidative stress, parturition, gestation, prematurity, membrane premature rupture

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INTRODUCTION

Telomeres are a highly conserved system that plays a central role in maintaining the integrity of the genome and cell. In somatic cells, telomeres reach a critical short length over the lifespan, or under the influence of stressors. Therefore, telomere length (TL) is an important feature of cell aging or senescence, which suggests the idea of a "biological clock," or a marker of cell replication (Hayflick, 1965; Blackburn, 2001).

In pregnancy, senescence has been related to term delivery mechanisms (Menon et al., 2014a; Behnia et al., 2015; Gomez-Lopez et al., 2017). The concept of placental cell aging in term delivery was first proposed in the 1970s; however, only lately has a relatively large number of studies examined the relationship between cell senescence and the consequent morphological changes in pregnancy. Placenta and fetal membranes constitute temporary tissues in the maternal body, and therefore, are "aged" and ready to be eliminated by the time of the term neonate is born. Thus, cellular senescence may be related to gestational complications if the process is activated prematurely (Behnia et al., 2015; Menon, 2016; Cox and Redman, 2017; Arias-Sosa, 2018). One of the main characteristics of senescent cells is the production of inflammatory cytokines, which indicates a possible role of cellular senescence as an effector pathway that converges to trigger parturition. Intrinsic and extrinsic stressors that induce variation in prenatal exposures and maternal states and conditions, such as cigarette smoking, air pollution, diabetes, obesity, oxidative stress, and inflammation are associated with cellular aging, evidenced by the shortening of telomeres in fetal cells (Whiteman et al., 2017). Thus, characteristics of maternal health status and behaviors during pregnancy may influence the individual's susceptibility or propensity to disease in later life. However, few studies have shown a direct correlation between telomere dysfunction specifically in fetal membranes and fetal or developmental programming.

Thus, we begin this review with a brief overview of telomere structure and functions, followed by a description of telomere-related senescence mechanisms and the role of telomere dynamics in pregnancy. We then proceed to discuss the findings regarding fetal membranes telomere-senescence-mediated parturition and adverse gestational outcomes. We conclude by summarizing current knowledge blanks and future research directions.

TELOMERES: STRUCTURE AND FUNCTIONS

Telomeres are nucleoprotein structures at the end of chromosomes that play a vital role in maintaining genomic stability and protecting the chromosomes against fusion and degradation (Blackburn, 1991; Blackburn et al., 2015). In humans, telomeres consist of 2–20 kb of non-coding double-stranded DNA formed by a conserved hexameric (TTAGGG) tandem repeat DNA sequence and a 3' overhang of the G-rich strand, which folds back into the double-stranded DNA, forming a structure known as the *t*-loop, important for protecting the genome from nucleolytic degradation, unnecessary recombination, repair, and interchromosomal fusion (Lu et al., 2013; Blackburn et al., 2015). Besides having a unique DNA sequence, the protection of chromosomes depends on the association and interaction of human telomeres with the shelterin complex, which contains six specific proteins (TRF1, TRF2, POT1, TIN2, TPP1, and RAP1). This network provides a compact chromatin structure that limits the accessibility of DNA damage repair (DDR) machinery and decreases its mistaken recognition at the telomere region (Bandaria et al., 2016; Fathi et al., 2019) (**Figure 1A**). Since DNA polymerase is unable to fully replicate the 3' end of the DNA strand, telomeres lose part of its sequence with each cell division and reach a critical short length, which, in turn, leads to cellular senescence (Stewart et al., 2003).

Stem cell compartments and embryonic stem cells present telomerase activity; this is a ribonucleoprotein complex, composed of telomerase reverse transcriptase (TERT) and rRNA telomerase component (TERC) subunits and serves as a template for the addition of telomeric repeats to chromosome ends. However, TERT expression and telomerase activity are often very low or undetectable in somatic cells, which explain, in part, the limited capacity of somatic cells to replicate (Rubtsova et al., 2012). Other pathways, such as the alternative lengthening of telomeres (ALT), have been reported in cancer cells (De Vitis et al., 2018). ALT is still not a well-known process, but it is related to telomeric recombination and may be activated when telomerase is repressed. Nonetheless, cultured cells over passages show limited replication capacity, which has been attributed mainly to the shortening of telomeres (Burton and Krizhanovsky, 2014).

Cell aging is a physiological process, as telomeres undergo steady attrition during the proliferation of normal cells; this can be either beneficial or detrimental to the organism. On one hand, it contributes to tumor suppression, limiting tissue damage, and possibly embryonic development, while on the other, it may be associated with aging-related diseases, impaired tissue regeneration, and cellular dysfunction, as well as pregnancy complications (Howcroft et al., 2013; Burton and Krizhanovsky, 2014; Menon et al., 2016; Arias-Sosa, 2018).

TELOMERE-RELATED SENESCENCE MECHANISMS

Replicative senescence is characterized by cellular proliferative capacity, which depends ultimately on progressive telomere shortening to a critically short length, responsible for the limited number of cell divisions (Bekaert et al., 2005). Therefore, TL is one the main inducers of cell aging or senescence, which suggests the idea of a "biological clock," or "Hayflick limit," first described in human fibroblasts cultured *ex vivo* as a proliferative limitation on cells, despite their viability (Hayflick, 1965; Campisi and D'Adda Di Fagagna, 2007; Xu et al., 2013).

The main telomere-related senescence mechanisms include DNA structure dysfunction and modifications. Chromatin and histones structure dysfunction is a key point associated with telomere shortening, which can be triggered by diverse pathways. First, a decrease in histone levels has been observed

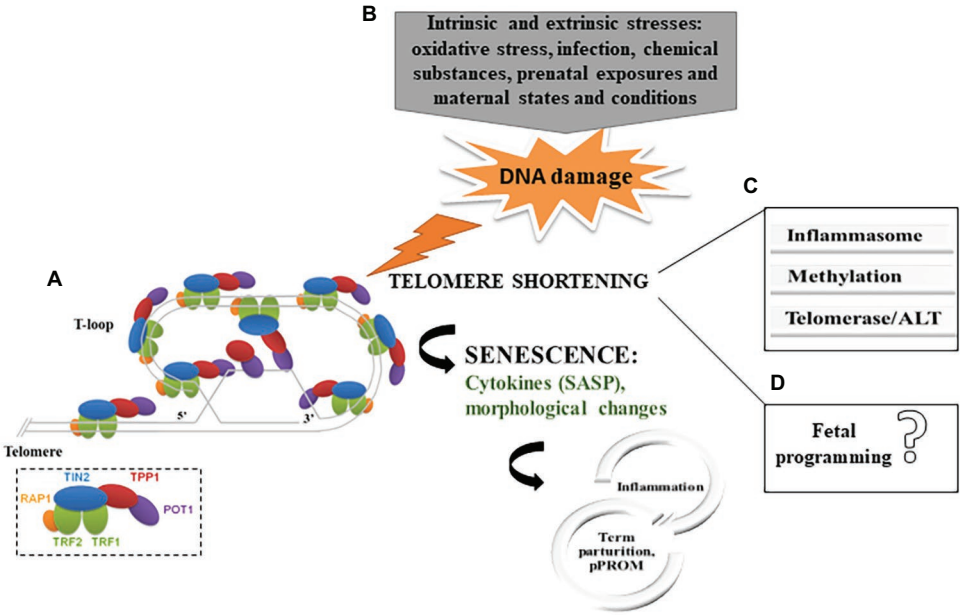

FIGURE 1 | (A) Telomere structure and telomere-binding proteins. The six shelterin proteins are depicted in the small box (Adapted from Lu et al., 2013). **(B)** Summary of the proposed mechanism for telomere shortening in fetal membranes in parturition. **(C)** Potential mechanisms of telomere-senescence-mediated parturition and adverse gestational outcomes in fetal membranes. **(D)** No evidence that telomeres dysfunction in fetal membranes has a direct effect on fetal programing (SASP, senescence-associated secretory phenotypes; ALT, alternative lengthening of telomeres; and pPROM, preterm premature rupture of membranes).

in human senescent fibroblasts compared to levels in younger cells, impairing processes such as replication, transcription, and DNA repair (O'Sullivan et al., 2010; Adams et al., 2013). Moreover, the majority of lysines on histones are physiologically hypoacetylated near telomeres, contributing to the genomic silencing of this region; thus, any histone modifications, such as acetylation, may interfere with the *t*-loop telomere structure. Such modification may also repress hTERT expression in human cells, such that senescence signaling is initiated (Cong and Bacchetti, 2000). Equally, changes in the structural and epigenetic integrity of telomeres throughout population doubling have an impact on core histones and their chaperones, which, in turn, ultimately lead to senescence (O'Sullivan et al., 2010).

Additionally, chromatin structure is also determined by DNA methylation and, although telomere sequence do not contain genes or CpG sequences, the subtelomeres regions (transition regions between the terminal telomeric repeats and the chromosome-specific regions) are notably CpG-rich, therefore, more prone to be physiologically highly methylated (Toubiana and Selig, 2020). Previous studies have shown that aberrant methylation of subtelomeric DNA exists in many diseases, and it has an impact on the TL regulation, as shorter telomeres are significantly associated with decreased methylation levels at most of CpG sites (Buxton et al., 2014; Hu et al., 2019). Changes in the methylation status of different CpG sites are typical in cancer cells (Joyce et al., 2018), but they have also been observed in senescent cells. This has been used by researchers to successfully predict the age of several different tissues and predispositions to aging-related diseases (Bell et al., 2019). This tool using a set of CpG sites is named epigenetic clock, which starts during development when fetal tissues, embryonic, and induced pluripotent stem cells reveal a DNA methylation age (DNAm age) (Horvath, 2013; Bell et al., 2019).

Besides replicative senescence, *stress-induced senescence* demonstrates that the Hayflick limit is no longer a constant but can vary depending on influencers of telomere loss, such as oxidative damage and/or decrease in antioxidative defense. Thus, the mechanisms described above can be influenced by stressors and accelerate the cell aging process. The accumulation of intrinsic and extrinsic stresses is a well-known pathway that triggers telomere dysfunction and impairs telomere end replication (Pickett and Reddel, 2012; Tan and Lan, 2017), mainly through oxidative stress (OS) (von Zglinicki, 2002; Tan and Lan, 2017).

Under conditions of genomic stability, DNA damage activates DDR complex that coordinates repair and cell cycle progression. Since the telomere is a guanine-rich region (triple structure), it is more vulnerable to OS damage compared to the general genome (von Zglinicki et al., 2000; Stewart et al., 2003; Xu et al., 2013); therefore, telomere dysfunction ultimately leads to cell cycle arrest (Rossiello et al., 2014). In mammalian cells, there are two main DDR mechanisms that address double strand breaks (DSBs): homologous recombination (HR) and non-homologous end joining (NHEJ); the latter is related to telomeric DNA. It has been suggested that NHEJ is inhibited by TRF2 shelterin, preventing chromosomal fusions, and, therefore, end-to-end fusion. Conversely, NHEJ is also the main mechanism for DNA

ligase *N*-dependent chromosomal fusions that occur between uncapped telomeres, which suggests a selective regulatory switch, from preventing recombination to promoting it (Evans and Cooke, 2007). OS is known to accelerate telomere attrition *in vitro* and *in vivo*. Pineda-Pampliega et al. (2020) described how oxidative stress shortens telomeres in free-living white stork chicks. According to the authors, the administration of antioxidants had a functional effect on oxidative stress. Furthermore, environmental and behavioral stressors were found to induce OS-induced telomere damage. Exposure to different organic pollutants such as dioxins, furans, and polychlorinated biphenyls (PCBs) through food, water, and air, which occur during the human lifetime, may change TL in peripheral blood leukocytes (Shin et al., 2010; Mitro et al., 2016; Karimi et al., 2020). Serum levels of organochlorine pesticides can be associated with oxidative stress and systemic inflammation that lead to telomere shortening (Karimi et al., 2020). The cellular exposure to non-ortho PCBs and toxic equivalency was associated with increased leukocyte TL in a study population of American adults, contributing population-level findings to the evidence that exposure to environmental contaminants may influence telomere regulation (Mitro et al., 2016). In this same direction, Shin et al. (2010) analyzed the impact of low-dose exposure to persistent pollutants, i.e., lipophilic xenobiotics, on the TL of peripheral blood leukocytes in healthy persons. It was concluded that TL increases with low doses of exposure, suggesting that low doses may act as tumor promoters in carcinogenesis in humans.

Therefore, how does oxidative stress cause telomere shortening? Although there are many suggested pathways for answering this question, the increase of reactive oxygen species and/or decrease in the antioxidant capacity mainly lead to damage in cellular structures, mostly inducing oxidized base in the DNA and consequent DDR defects. Kawanishi and Oikawa (2004) have reported DNA damage caused by the treatment of fibroblast with UVA irradiation, including 8-hydroxy-2'-deoxyguanosine (8-OHdG) formation, specifically at the GGG sequence in the telomere sequence, which was correlated with a decreased in TL. The enzyme responsible for repairing this DNA damage is an 8-oxoG-DNA glycosylase (OGG1), and its action begins by excising the damaged base and subsequent replacement of the modified base (Rosenquist et al., 1997). This local DNA damage can disrupt cell replication; consequently, if the repair mechanism by OGG1 is impaired, a damage to single-stranded DNA strand in the telomere region occurs, which contributes to its shortening. Under the repair failure, mechanisms of DDR by sensor proteins such as the ataxia telangiectasia mutated (ATM) kinase is activated, which regulates the early step(s) of DNA damage signaling, and thereby controls DDR. Persistent DNA damage in response to overwhelming OS causes DNA breaks followed by the phosphorylation of the histone H2AX (γ-H2AX). This *via* induces mechanism of cellular damage involving p53 activation, as well described in cells such as fibroblasts. Alternatively, the route that leads to senescence can be p53-independent, through the activation of mitogen-activated protein kinases (MAPKs) pathway (Iwasa et al., 2003). Salminen et al. (2012) have reported either pathway converges to a downstream activation of NF-κB signaling. In turn, NF-κB system is linked to inflammatory responses in cellular senescence. It is important to note that senescent cells acquire many changes in gene expression, resulting in changes in secreted proteins, such as growth factors, proteases, chemokines, and cytokines, that, together, characterizes the senescence-associated secretory phenotype (SASP; Davalos et al., 2010; Freund et al., 2010; Rodier and Campisi, 2011) (**Figure 2**).

Additionally, a newly discovered telomere-related stressor is sex hormone concentrations, but a direct relation between sex hormones and TL, if any, remains uncertain. Preliminary evidence suggests that sex steroid hormones could be involved in enhancing telomerase activity since serum dihydrotestosterone and estradiol are positively correlated with leukocyte TL independently of age (Yeap et al., 2016, 2020). However, other studies have demonstrated no association of short TL with sex hormones in healthy men and women (Coburn et al., 2018; Gu et al., 2020).

As a result of telomere-related OS disorders, some pathological conditions have been described, including diabetes and vascular disease (Blackburn et al., 2015). Primary cultures of fibroblasts were used to examine the impact of a diabetic environment on telomeres and, under elevated glucose conditions, relative TL loss was observed in this model (Sutanto et al., 2019). On the other hand, type 2 diabetes mellitus patients with non-alcoholic fatty liver disease have a significantly longer leukocyte TL than patients without non-alcoholic fatty liver disease (Zhang et al., 2019). Telomere shortening has been associated with premature vascular aging, which may be involved in lower-extremity amputation in patients with type 1 diabetes at high vascular risk (Sanchez et al., 2020). Of particular interest here, telomere dysfunctions are associated with placental aging in the etiology of parturition and adverse pregnancy outcomes, in which fetal membranes and gestational tissue play a crucial role.

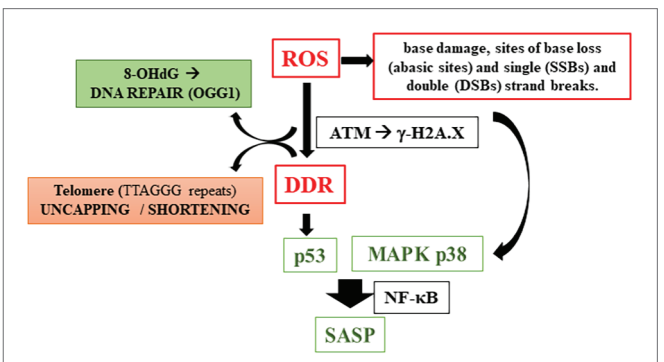

FIGURE 2 | Reactive oxygen species induction of telomere shortening (8-OHdG, 8-hydroxy-2'-deoxyguanosine; OGG1, 8-oxoG-DNA glycosylase; ATM, ataxia telangiectasia mutated kinase; DDR, DNA damage response; MAPK, mitogen-activated protein kinases; and SASP, senescence-associated secretory phenotypes).

FETAL MEMBRANES TELOMERE-SENESCENCE IN PARTURITION AND ADVERSE GESTATIONAL OUTCOMES

In recent years, new evidences have shown that cell senescence is related to term delivery mechanisms (Menon et al., 2014b; Behnia et al., 2015; Gomez-Lopez et al., 2017). The concept of placental cell aging in term delivery was first proposed in the 1970s (Rosso, 1976); however, until recently few studies had reported on cellular senescence related to oxidative stress and its consequent morphological changes (Menon et al., 2012; Polettini et al., 2015b). This process is believed to be physiological since the placenta and fetal membranes constitute temporary tissues in the maternal body, and therefore, would be "aged" and ready to be eliminated after the birth of the neonate (Behnia et al., 2015; Menon, 2016; Cox and Redman, 2017; Arias-Sosa, 2018).

Recent investigations have revealed that term pregnancies are characterized by increased OS that induces DNA damage (Menon, 2016; Cox and Redman, 2017; Arias-Sosa, 2018); therefore, TL is affected. Gestational tissues show evidence of senescence, given that the telomere attrition rate is negatively correlated with gestational age, thus the closer to term, the shorter the telomeres in fetal and placental cells (Gielen et al., 2014; Casavant et al., 2019). Accordingly, recent findings have demonstrated that fetal membranes from term in labor pregnancies had shorter TL than both preterm and term not in labor pregnancies, suggesting the senescence of term placentas along with labor (Menon et al., 2012; Polettini et al., 2015a; Colatto et al., 2020). The same results were observed in fetal membranes and placenta in mouse (Phillippe et al., 2019), providing support for the hypothesis that shorter telomeres at term potentially function as a biologic clock for parturition.

Besides TL analysis, term labor tissues show features and markers of senescence, such as histological enlarged cells and organelles, granulated nuclei, and more intense staining of senescence-associated β-galactosidase (a lysosomal enzyme; Menon et al., 2014a; Behnia et al., 2015) that strengthens the indication of fetal membranes senescence by parturition time. Among the changes in senescent cells, the production of inflammatory mediators is of particular interest (Freund et al., 2010), as they induce parturition. Fetal membranes play a crucial role in this process, as these tissues are in close contact with the amniotic fluid and, consequently, the fetus (Parry and Strauss, 1998). In the third trimester of pregnancy, chorioamniotic cells increase their production of mediators, especially pro-inflammatory cytokines, immunomodulatory cytokines, neutrophil recruitment chemokines, and arachidonic acid metabolites (Kamel, 2010; Hua et al., 2013; Romero et al., 2018). These mediators are essential to stimulate the production of prostaglandins and consequent uterine contractility (Romero et al., 2006).

Therefore, as is well-known, inflammation is a key point in parturition, even in the absence of intrauterine infection, and, in such cases, inflammation may come from the senescence of fetal membranes (Behnia et al., 2016; Martin et al., 2017). Behnia et al. (2015) found higher concentrations of pro-inflammatory SASP markers (granulocyte macrophage colony-stimulating factor and interleukin-6 and -8) in the amniotic fluid of women in labor at term than in women not in labor. Additionally, bioinformatics analysis has shown, under both term and preterm conditions, that maternal exosomes (30–150 nm particles that propagate to distant sites) carry proteins associated with inflammatory and metabolic signaling (Menon et al., 2019). Besides cellular alterations to the pro-inflammatory profile, the inflammasome might be activated during the parturition process by senescent cells with shortened telomeres. Recent findings have revealed telomere dysfunction as a cause of macrophage mitochondrial abnormalities, OS and hyperactivation of the NOD-like receptor family pyrin domain-containing protein 3 (NLRP3) inflammasome (Kang et al., 2018). Likewise, fetal membranes from women who underwent term labor had higher concentrations of NLRP3 (Romero et al., 2018); thus, dysfunctional telomeres might work as a primary factor and cooperate to amplify inflammasome signaling related to parturition signaling. Crosstalk between these pathways may prove to be a key molecular mechanism of immunosenescence that has been reviewed elsewhere (Jose et al., 2017; Ventura et al., 2017) (**Figure 1B**).

In this context, telomere-related cellular aging may be linked to gestational complications if the process is activated prematurely (Biron-Shental et al., 2010; Menon et al., 2012; Smith et al., 2013). Adverse pregnancy outcomes, such as stillbirth, intra uterine growth restriction, and preeclampsia are related to trophoblast dysfunction and attributed to placental villous telomere shortening (Biron-Shental et al., 2014; Ferrari et al., 2016; Paules et al., 2019). As mainly of these adversities are linked to placental dysfunction, the role of senescence in fetal membranes has been poorly investigated in these complications.

One of the firsts reports in fetal membranes demonstrated shorter TL from preterm premature rupture of membranes (pPROM) compared to preterm labor (PTL) with intact membranes pregnancies at the same gestational age, and the first was similar to term (Menon et al., 2012). More recently, structural and histological changes in fetal membranes from women undergoing pPPROM were found to be compatible with senescence, suggesting its role in disrupting membranes remodeling and homeostasis, with overwhelming ROS-associated inflammation (Menon et al., 2014a; Behnia et al., 2015; Menon, 2016; Menon and Richardson, 2017). Mechanistically, telomere shortening in pPROM is likely a result of senescence activators, such as MAPKs, that are increasingly expressed in fetal membranes in pregnant women with pPROM (Lappas et al., 2011). Dutta et al. (2016) have reinforced that prosenescence stress kinase (p38MAPK) activation, OS damage, and signs of senescence are pronounced in fetal membranes from pPROM in comparison with PTL with no rupture of membranes. Accordingly, the induction of OS caused significant protein peroxidation in the amniotic sac in mouse, which was associated with p38MAPK activation and senescence, in addition to increased concentrations of pro-inflammatory cytokines in amniotic fluid (Polettini et al., 2018). Commonly, OS activates a specific p53 transcriptional response in diverse tissues, which regulates the cellular

response to DNA damage; however, fetal membranes fail in activating p53 under OS, suggesting a diverse pathway triggering senescence in this tissue (Polettini et al., 2015a) (**Figure 1B**).

Important to note that stressed and injured cells and tissue release stimulatory molecules, such as host-derived damage-associated molecular patterns (DAMPs), that signaling through toll-like receptors (TLRs) and activates cellular response. Therefore, telomere shortening in fetal membranes may also provide additional signs to initiate parturition. In senescent fetal membranes, two DAMPs have been reported, the high mobility group box 1 (HMGB1) and cell-free fetal telomere fragments (Bredeson et al., 2014; Polettini et al., 2015a). Detection of circulating nucleic acids in maternal plasma and serum, such as cell free fetal DNA, has emerged as a predictor marker or monitoring tool for the most common and severe pregnancy complications (Phillippe, 2015). One hypothesis is that DNA telomere fragments from senescent amnion cells are shed into the amniotic fluid, and these fragments can accelerate senescence in healthy gestational tissue, as a fetal signal at term that can cause labor-associated changes (Polettini et al., 2015a). Such observation has been supported by recent *in vitro* experiments, as amniotic cells under OS produce exosomes packed with fetal telomere fragments (Sheller-Miller et al., 2017). The recognition of these molecules was speculating to be through TLR-9 that is known to trigger maternal immune cells activation in response to placenta-derived DNA (Hahn et al., 2014). However, no difference in TLR-9 expression was observed in amnion cells treated with telomere fragments compared to controls (Polettini et al., 2015a). Thus, further experiments are needed to address the specific mechanism by which telomere fragments activate intracellular signaling in fetal and maternal cells.

Regarding methylation, chorionic villi, maternal decidua, fetal membranes, and embryonic tissues have a unique DNAm setting (Robinson and Price, 2015). Particular alterations in DNAm signatures were observed in placentas and fetal membranes with acute chorioamnionitis (Konwar et al., 2018). Moreover, many investigators have described cord blood DNAm related to prematurity and inflammation (Liu et al., 2013; de Goede et al., 2017), but data on telomere-associated methylation are scarce. Wilson et al. (2016) found a correlation between shorter TL and decreased DNAm in genes associated with the telomerase regulation in placentas. The authors have described that almost 20% of the probes within TERT gene showed significant alterations in DNAm associated with TL, but they discuss epigenetic regulation of the TERT is complex, and such changes should be observed throughout the entire TERT gene region in order to elucidate the biological relevance of DNA methylation in this region. In fetal membranes, epigenetic modifications have been described, such as non-coding RNA (lncRNA) that has also been linked to pPROM (Luo et al., 2013). However, as our knowledge, there are yet no studies that have demonstrated telomere-related methylation in fetal membranes and the possible association with parturition and gestational outcomes.

Additionally, low levels of telomerase activity have been associated with TL reduction in the placentas of babies with delayed fetal development in term pregnancies, attributed to accelerated telomere DNA loss and cellular senescence (Davy et al., 2009). Fetal membranes, in particular, maintain characteristics of pluripotent cells; therefore, it would be expected to find telomerase activity in such tissues (Zhou et al., 2013). However, potency, cellular transition capability, and migratory potential are lost in fetal membranes as gestation progresses and/or in response to OS-inducing factors (Richardson and Menon, 2018), and low telomerase activity was detected in fetal membranes regardless term or PTL (Colatto et al., 2020). Accordingly, amniotic fluid derived cells, including amniotic cells, also lack telomerase activity (Chen et al., 2013). Alternatively, ALT can be activated when telomerase is suppressed; however, to date, ALT activity has not been investigated in gestational tissues. ALT regulation is influenced by telomeric repeat-containing RNA (TERRA), a lncRNA, which works as a telomerase-telomere binding inhibitor. In turn, TERRA expression is directly controlled by DNA methylation at the CpG rich gene promoters (Nabetani and Ishikawa, 2011; Coluzzi et al., 2017). In placentas, Novakovic et al. (2016) have demonstrated higher TERRA expression compared to matched somatic cells from cord blood, which was correlated to very low levels of hTERT in first trimester cytotrophoblasts. These data suggest that additional pathways might be involved in TL regulation other than telomerase activity in fetal membranes, but this requires further investigation.

DISCUSSION

A vast literature demonstrates that telomere-dependent replicative senescence in placental and fetal membranes is involved with parturition and gestational adversities. We have summarized that OS, inflammation, methylation, telomerase, and ALT are the main described mechanisms in telomere biology in fetal membranes to date (**Figure 1C**). Diverse DNA methylation are detectable for most cancer-CpG sites beginning 4 years of pre-diagnosis (Joyce et al., 2018); thus, similarly, the understanding of telomere biology, shortening of TL mechanisms, and methylation regulation in fetal membranes might provide early evidences during gestational periods in relation to telomere alterations and the propensity for pregnancy outcomes. Moreover, telomerase and ALT regulation are not fully understood in fetal membranes, which reinforces that further investigation is needed regarding telomere dysfunction in fetal membranes.

The mean TL set during intra uterine development likely has an impact on later extrauterine life, as variation in prenatal exposures and maternal states and conditions may impact fetal developmental trajectories. Subsequently, fetal or developmental programming may influence an individual's susceptibility or propensity for disease in later life (Entringer et al., 2018). A recent systematic review demonstrated that maternal factors such as age, exposure to chemicals (e.g., smoking), and maternal stress during pregnancy and nutritional and sleep disorders are related to the stimulation of telomeres in fetal cells (Whiteman et al., 2017). Also, it has been documented that if TL is reduced in the newborn, the susceptibility to the development of chronic diseases in adulthood is increased (Entringer et al., 2012).

Thus, in terms of fetal programming of the telomere system, the maternal-placental-fetal immune activation, characterized by the increased expression of pro-inflammatory cytokines in response to various adverse conditions during pregnancy, may have the potential to impact fetal TL. Although the maternal and the environmental exposure during the intrauterine period is correlated to the postnatal period, as well as with the outcomes of newborn infants, there is lack of such relation in fetal membranes studies and more studies are required to understand whether telomeres dysfunction in fetal membranes has a direct effect on fetal programing (**Figure 1D**).

Unfortunately, the ability to mediate a delay in parturition during pregnancy complications is limited, and the development of successful effective interventions requires a more complete understanding of the molecular mechanisms that trigger parturition, including telomere signals, which may vary throughout placental tissues.

AUTHOR CONTRIBUTIONS

JP and MGS have equally contributed to this review and approved the submitted version.

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REVIEW
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Fetal Membrane Epigenetics

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The characteristics of fetal membrane cells and their phenotypic adaptations to support pregnancy or promote parturition are defined by global patterns of gene expression controlled by chromatin structure. Heritable epigenetic chromatin modifications that include DNA methylation and covalent histone modifications establish chromatin regions permissive or exclusive of regulatory interactions defining the cell-specific scope and potential of gene activity. Non-coding RNAs acting at the transcriptional and post-transcriptional levels complement the system by robustly stabilizing gene expression patterns and contributing to ordered phenotype transitions. Here we review currently available information about epigenetic gene regulation in the amnion and the chorion laeve. In addition, we provide an overview of epigenetic phenomena in the decidua, which is the maternal tissue fused to the chorion membrane forming the anatomical and functional unit called choriodecidua. The relationship of gene expression with DNA (CpG) methylation, histone acetylation and methylation, micro RNAs, long non-coding RNAs and chromatin accessibility is discussed in the context of normal pregnancy, parturition and pregnancy complications. Data generated using clinical samples and cell culture models strongly suggests that epigenetic events are associated with the phenotypic transitions of fetal membrane cells during the establishment, maintenance and termination of pregnancy potentially driving and consolidating the changes as pregnancy progresses. Disease conditions and environmental factors may produce epigenetic footprints that indicate exposures and mediate adverse pregnancy outcomes. Although knowledge is expanding rapidly, fetal membrane epigenetics is still in an early stage of development necessitating further research to realize its remarkable basic and translational potential.

Keywords: amnion, chorion, decidua, chromatin modifications, non-coding RNAs, human pregnancy, parturition

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INTRODUCTION

Current consensus defines an epigenetic trait as "a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence." (Berger et al., 2009) The fetal membranes and the adjacent decidua adopt a phenotype in early gestation that supports pregnancy by preserving the integrity of the gestational sac, reducing myometrial contractility and controlling maternal innate and adaptive immunity to tolerate the semi-allogenic fetus. These protective characteristics are maintained stably throughout pregnancy despite the massive growth

of the gestational sac. At term, a phenotype transition occurs that promotes membrane rupture, myometrial contractions, inflammation and lowered immune tolerance (Menon et al., 2016, 2019). The changes trigger birth. Similar changes can be elicited by pathological conditions, such as genital tract infection, pre-eclampsia and uterine overdistension, often inducing birth before term (Romero et al., 2014).

Epigenetic events establish, sustain and adjust chromatin structure in a cell-specific fashion through DNA methylation, post-translational histone modifications and regulatory non-coding RNAs. The resulting chromatin landscapes determine cell-specific gene expression patterns, which determine tissue phenotypes (Roadmap Epigenomics Consortium et al., 2015). Evidence is accumulating that characteristic chromatin modification patterns and non-coding RNA transcriptomes occur in the fetal membrane and decidua cells and change dynamically during normal and pathological pregnancies. This suggests that the mechanisms driving the phenotype transformations during gestation and at birth are, at least partially, epigenetic. This article summarizes information about epigenetic processes and associated phenotype changes in the amnion, chorion laeve and the decidua and consider their significance in normal pregnancies, during labor and in pregnancy disorders.

AMNION

In primates, amnion epithelial cells differentiate from epiblasts at about Day 8 of pregnancy, which is prior to gastrulation (Enders et al., 1986; Sasaki et al., 2016). The pluripotent epiblast phenotype is preserved throughout pregnancy to a substantial degree, as evidenced by the ability of amnion cells to differentiate to cells of all three germ layers *in vitro* under appropriate conditions (Easley et al., 2012). During the most of the gestation, the amnion exhibits characteristics that are anti-inflammatory and smooth muscle relaxant in agreement with a role in protecting the pregnancy (Carvajal et al., 2006; Silini et al., 2013). Furthermore, the mechanical strength of the gestational sac is provided by the strong collagenous ("compact") layer of the amnion membrane, which is maintained by the fibroblastic (mesenchymal) cells of the connective tissue underlying the epithelial layer. There is now evidence that the mesenchymal cells may be derived from the epithelial cells that undergo reversible epithelial-to-mesenchymal cell transformation (EMT) allowed by their phenotypic plasticity. There is also evidence that EMT in the amnion occurs increasingly with advancing gestation and in response to the proinflammatory cytokine, TNFα, or oxidative stress, which intensifies remodeling and results in the mechanical weakening of the membrane (Janzen et al., 2017; Richardson et al., 2020). Moreover, amnion mesenchymal cells respond strongly to proinflammatory stimuli, further promoting membrane rupture and the production of uterotonic factors including prostaglandins (Whittle et al., 2000; Sato et al., 2016). Thus, controlled and properly timed phenotype transitions of the amnion cells are critical for both maintaining pregnancy and triggering birth. Epigenetic events impacting on gene expression patterns are believed to contribute to the gestational transformations of amnion cells, which is stimulating interest in the topic.

DNA Methylation

Methylation of cytosines at the 5th position in the CpG motifs of DNA (5meCpG) is the most thoroughly studied epigenetic chromatin modification. CpG methylation is traditionally considered to silence genes by promoting closed chromatin structure or recruitment of repressor complexes to gene regulatory regions (Jones, 2012). There are about 28 million CpG sites in the human genome and more than 80% of these are methylated (Breiling and Lyko, 2015; Lövkvist et al., 2016). The density of DNA methylation in particular chromatin regions depends on the frequency of CpG dinucleotides, which varies substantially in the genome. Recent global analyses of CpG methylation levels have indicated, however, that the relationship of DNA methylation to gene activity depends on the genomic context. For example, higher CpG frequency regions, called CpG islands (Gardiner-Garden and Frommer, 1987), are generally unmethylated in promoters, and CpG sites are highly methylated in the transcribed regions of active genes. Low CpG density promoters and regulatory regions may exhibit variable methylation associated with variable gene activity, which is often tissue-specific and change with cellular differentiation (Jones, 2012).

Although DNA CpG methylation is considered a stable and mitotically heritable epigenetic modification, it undergoes turnover catalyzed by DNA methylating and demethylating enzyme systems (Schubeler, 2015; Kim and Costello, 2017). DNA methylation in human cells is performed by a family of DNA methyl transferases (DNMTs), which includes DNMT1, DNMT3A, -3B, and DNMT3L. DNMT1 is generally responsible for "maintenance" methylation during the S- (DNA-replicating) phase of the cell cycle because of its selectivity toward hemi-methylated CpG motifs in the nascent double-stranded DNA. DNMT3A and -3B perform "*de novo*" methylation at unmethylated CpG sites. DNMT3L is a catalytically inactive essential cofactor of DNMT3A and -3B. The characteristics of DNA methyltransferases have been extensively studied and reviewed in the literature (Tajima et al., 2016; Gowher and Jeltsch, 2018). DNMTs (except for DNMT3L) are expressed in the amnion and the decidua (Grimaldi et al., 2012; Mitchell et al., 2013) and are discussed in later sections. A group of 5meCpG-binding proteins (MBDs) recognize and functionally interpret DNA methylation patterns as signals for gene repression in most genomic contexts (Du et al., 2015). The mechanisms include heterochromatin formation, establishment of repressive histone modifications, nucleosome remodeling and extension of methylated DNA regions. Of the 11 MBD proteins identified so far (Du et al., 2015) one, MBD5, has been reported in decidualizing endometrial cells (Grimaldi et al., 2012). MBD proteins and their roles as methylated DNA readers is a major unexplored area of fetal membrane epigenetics.

The elucidation of the biochemical mechanisms that erase the 5meCpG modification was challenging because of the large energy barrier obstructing the direct enzymatic removal of the

5-methyl group. The best characterized removal pathway is initiated by the oxidative modification of the 5-methyl group producing 5-hydroxymethyl cytosine (5-hmC). The reaction is catalyzed by the Ten-Eleven Translocation group of dioxygenases (TET-1, -2, and -3), which use molecular oxygen and 2-oxoglutarate as co-substrates. TETs can oxidize 5-hmC further to 5-formyl and 5-carboxyl cytosine, which are detected by the base excision DNA repair (BER) system eventually replacing 5meC with unmodified cytosine in the CpG motifs (Kohli and Zhang, 2013; Bochtler et al., 2017). The central role of 5-hmC in DNA demethylation is reinforced by the inefficiency of DNMT1 to recognize 5-hmCpG for maintenance methylation, which results in the "passive" loss of methylation of the affected 5meCpGs during replication (Hashimoto et al., 2012; Kohli and Zhang, 2013). Thus, 5-hmC is generated from 5meC and as a consequence its incidence is lower than that of 5meC in somatic cells (Globisch et al., 2010). Its presence, however, indicates sites of dynamic DNA methylation in the genome such as poised enhancers, low- and intermediate CpG density promoters and bivalent promoters that are subject to developmental regulation (Yu et al., 2012; Gao and Das, 2014). Finally, proteins that bind 5-hmCpGs with high affinity and may function as epigenetic readers of 5-hmCpG and its oxidized derivatives have been discovered (Spruijt et al., 2013) suggesting that these modified bases may carry epigenetic information in addition to their role in 5meCpG turnover.

DNA methylation in the fetal membranes has been studied so far using traditional techniques that do not discriminate between 5meC and 5-hmC such as bisulfite conversion (Huang et al., 2010) or have no verified selectivity between the two modified bases (e.g., enzyme-based assays). These studies are mostly descriptive, and the results are reported in terms of CpG methylation. The main findings are summarized and discussed in the following sections.

Genome-Wide Profiling

CpG methylation has been profiled genome-wide in the amnion using the Illumina Infinium HumanMethylation27k BeadChip (HM27k) array (Eckmann-Scholz et al., 2012). The array features probes targeting over 25,000 CpG sites preferentially in promoter CpG islands with approximately 2–3 sites per gene. The probed CpGs showed bimodal distribution with either high or low levels of methylation in cells isolated from mid-trimester amniocentesis samples and expanded in culture. Such distribution is expected in somatic cells (Pidsley et al., 2016). Methylation patterns distinguished amnion-derived cells and villous chorion samples at similar gestational age as determined by principal component analysis (PCA) and hierarchical clustering (Eckmann-Scholz et al., 2012). However, amniotic fluid may contain cells from fetal skin, airways and intestine, potentially confounding the characterization of cells originating from amnion tissue. In another study (Kim et al., 2013), amnion tissue samples from women after term and preterm labor and term not in labor were processed for methylation analysis with the HM27k array. PCA analysis robustly separated the amnion samples according to the presence or absence of labor, but not according to gestational age. Nevertheless, more than 60 genes were found to be differentially methylated at term labor versus preterm labor and term not in labor versus term labor ($p < 0.0001$), with no overlap among the top 15 differentially methylated genes in the two comparisons. Yoo et al. (2018) used the more advanced HM450 BeadChip (HM450k) containing over 485,000 probes offering a much wider genomic coverage that includes CpG islands, shores and shelves, gene bodies, untranslated regions and enhancers (Pidsley et al., 2016). Amnion tissues after term and preterm delivery were compared and methylation differences were matched to differential gene expression determined by whole transcriptome sequencing. Nearly 36,000 differentially methylated CpG sites and over 1,000 differentially expressed genes were found, of which, 71 genes exhibited reciprocal changes of expression and CpG methylation in either direction. Two genes related to cell adhesion, integrin subunit alpha 11 (*ITGA11*) and trombospondin-2 (*THBS2*), were selected for verification using bisulfite pyrosequencing and real-time RT-PCR with independently collected samples. Both genes showed lower methylation and higher expression preterm than at term. Although this study used genome-wide discovery approaches to find previously discovered and expected relationships between gene methylation and expression, it has confirmed that CpG methylation in the amnion is dynamic and related to transcriptional activity at a subset of genes linked to tissue function.

Pre-eclampsia is a severe pregnancy complication characterized by hypertension, proteinuria and maternal inflammatory reactions. Its pathogenesis is unclear but abnormal placentation, angiogenic imbalance and endothelial dysfunction are well-documented attributes of the condition. DNA methylation has been studied in the early onset form (<34 weeks of gestation) of the disease using formaldehyde-fixed, paraffin-embedded full thickness fetal membrane samples that included amnion, chorion and attached decidua (Ching et al., 2014). CpG methylation profiling with the HM450k system showed nearly 10,000 differentially methylated CpG sites with mostly increased methylation in pre-eclampsia. Differentially methylated CpGs in gene-annotated genomic regions revealed decreased methylation in promoters and increased methylation within gene bodies, consistent with widespread transcriptional activation. A number of these genes and associated pathways have been found previously to be activated in pre-eclamptic placentae. Promoter hypomethylation has also been found in several pri-microRNA (pri-miRNA) genes, indicating the epigenetic regulation of miRNA expression. Thorough bioinformatic analysis has established the fetal membranes/decidua as epigenetic responders to the pre-eclamptic condition, but tissue-specific responses remained uncharacterized because of the use of unseparated full thickness membrane samples. Suzuki et al. (2016) addressed cellular heterogeneity in the amnion by performing whole genome bisulfite sequencing with separated amnion epithelial and mesenchymal cells. They found cell-specific methylation patterns and identified one CpG site (in an intron of the *SIPA1L1* gene) with a robust cell type-specific methylation difference, which could be used as a marker to correct for the variable cell type composition in amnion tissue samples. Genome wide methylation analysis was

conducted with an assay called HELP-tagging, which utilized the methylation sensitive restriction enzyme, *Hpa*II. With this assay the authors surveyed the methylation state of over 545,000 CpG sites in normal versus pre-eclamptic amnion samples (62, overall) and found 4,058 differentially methylated sites in 3,035 genes. Methylation of 123, 85, and 99 sites were influenced by systolic blood pressure, proteinuria grade and the combination of the two, respectively, in regression models. RNAseq with a subset of samples revealed that 41 genes were differentially expressed in pre-eclampsia; however, none of the differentially methylated sites were in the vicinity of the differentially expressed genes, indicating the complexity of 5meCpG-mediated gene regulation. Overall, the presence of pre-eclampsia-associated epigenetic "signatures" in the amnion is remarkable, because this tissue is not a prime player in the disease. It appears, however, that epigenetic plasticity makes the amnion a useful surrogate to report the effects of adverse intrauterine conditions on fetal tissues in general, which may impact on disease susceptibility later in life.

Candidate Genes

Methylation analysis of candidate genes having well-established functions is a straightforward approach to explore the role of DNA methylation in gene regulation with physiological relevance. Wang et al. (2008) examined the methylation of the *MMP1* gene promoter in the amnion in normal pregnancies and in cases of preterm pre-labor rupture of the membranes (pPROM). Matrix metalloproteases (MMPs), such as MMP1, have a key role in breaking down the collagen matrix of the amnion contributing to membrane rupture at term and preterm birth. The *MMP1* proximal promoter contains no CpG island, only 14 sporadic CpG sites, which are fairly highly methylated (≈50%) in amnion samples, as determined by clonal bisulfite sequencing. Treatment of primary cultures of amnion mesenchymal cells with the DNA-demethylating agent, 5-aza-3′-deoxycytidine (5-AZA), increased *MMP1* expression and decreased the methylation of one CpG motif at 1,538 bases upstream of the transcription initiation site. Interestingly, the methylation level of this site was significantly lower in amnion samples from deliveries complicated by pPROM compared to normal term controls. This group also described a previously undetected polymorphism in the *MMP1* promoter that generated a new CpG site as a minor allele. The presence of this CpG reduced *MMP1* promoter activity in amnion mesenchymal cells and even more so when it was methylated. *In vivo*, this site was always methylated and, remarkably, was significantly protective of pPROM in an African American population. This study illustrates that epigenetic and genetic differences may combine forming complex patterns of regulation that can be understood by analyses involving both levels. In the case of *MMP1*, the effect of DNA methylation on gene activity interacted with the polymorphism of the promoter shaping the impact on fetal membrane integrity and the risk of pPROM.

TIMP1 is a protease inhibitor protein that interacts with MMPs to control extracellular matrix remodeling. The *TIMP1* proximal promoter contains no CpG island and the 25 CpG sites around the transcriptional start site (−275)–(+279) are highly methylated in the amnion of female fetuses compared to males, as shown by Vincent et al. (2015), using the Sequenom Epityper technology. This is not surprising, since the gene is located in the X chromosome and one copy is in the hypermethylated homologous X chromosome in females. Notably, however, *TIMP1* mRNA abundance was higher in female than in male amnions and mRNA expression did not change with spontaneous labor despite a significant decrease of promoter methylation. Lipopolysaccharide (LPS) treatment of amnion explants increased methylation without effect on expression. Pre-treatment with the DNA demethylating agent, 5-AZA (5 uM, 48 h), did not affect *TIMP1* mRNA, or *TIMP1* promoter methylation levels in male or female amnions but sensitized the tissues to respond to LPS with increased *TIMP1* expression. These data suggest that *TIMP1* promoter methylation is dynamic but not linked to the level of gene activity in the amnion. Responsiveness to LPS might be influenced by methylation possibly at remote sites *in trans*.

Methylation of candidate genes in the amnion has been assessed in two further studies using the Methyl-Profiler PCR system (Mitchell et al., 2013; Sykes et al., 2015). The Methyl-Profiler (or MethylScreen) technology employs methylation-dependent and methylation sensitive restriction enzymes to probe the methylation density of pre-selected DNA sequences (Holemon et al., 2007). Labor-associated inflammatory genes (*PTGS2*, *BMP2*, *NAMPT/PBEF*, *CXCL2*), steroid receptor genes (*ESR1*, *PGR*, *NR3C1/GR*) and renin-angiotensin system components (*ACE*, *ATP6AP2/PRR*, *AGTR1*, *CTSD*, *KLK1*) were examined with the technique for promoter methylation density in amnion samples from early gestation (11–17 weeks) and after term delivery with or without labor. With the exception of *KLK1* (kallikrein 1), the proximal promoters of these genes have relatively high CpG density overlapping with CpG islands. Methylation densities of these promoters showed bimodal distribution with either highly methylated or sparsely methylated copies. The distributions did not change with gestational age or with labor but varied between genes and among individuals. Furthermore, the distribution of highly versus sparsely methylated promoter copies did not correlate with expression levels but correlated significantly between individuals. This suggests that the methylation of these promoters was established in early pregnancy (before 11–17 weeks) in a gene-specific fashion under the influence of individual conditions and was maintained until after delivery. In agreement with this, the expression of the DNMTs, DNMT1 and -3a, were highest in early pregnancy and decreased by term. Interestingly, the low CpG *KLK1* promoter exhibited intermediate methylation density in some of the samples and a loss of methylation at term, indicating dynamic methylation without a significant influence on expression level.

Histone Modifications

Covalent post-translational modifications of histones, which include acetylation, methylation, phosphorylation, ubiquitination, and sumoylation, are pervasive throughout the chromatin and are organized in patterns characteristic of genomic features, such as promoters, enhancers, transcribed

sequences and heterochromatin regions (Roadmap Epigenomics Consortium et al., 2015). They contribute to functional states such as open or closed chromatin, active or repressed genes, poised or operative enhancers. Recent evidence also indicates that modified histones are involved in directing DNA methyltransferases to chromatin regions where DNA methylation occurs during replication and cell differentiation (Fu et al., 2020). For example, DNMT3A and -3B contain PWWP domains that bind trimethylated lysine-36 in histone 3 (H3K36me3) (Rona et al., 2016), potentially directing these enzymes to exonic sequences of transcribed genes that are highly methylated and rich in H3K36me3. In addition, targeting of DNA methylation to heterochromatic regions through interactions between DNMT3A/B and methylated H3K9, or the lysine methyltransferase establishing this gene silencing modification (G9a), or chromodomain (methyl-lysine binding) proteins associating with methylated H3K9, has been reported but the molecular mechanisms are still unclear (Rose and Klose, 2014). Moreover, DNMT3 proteins contain ADD domains, which specifically recognize unmethylated H3K4 (H3K4me0), potentially explaining the antagonism between DNA methylation and H3K4 methylation genome wide (Fu et al., 2020). Even maintenance methylation by DNMT1 has been shown to depend on histone modifications such as H3K9 methylation and H3K27 ubiquitination [reviewed by Rose and Klose (2014)].

Despite their significance in epigenetic regulation, histone modifications are still scantly characterized in the fetal membranes. For example, genome-wide screens performed routinely using chromatin immunoprecipitation with antibodies selective for modified histones (ChIP-seq) have not been published with amnion or chorion to inform about gestational changes or pathological alterations. Studies employing ChIP combined with PCR, however, have demonstrated the presence of histone modifications in the promoter regions of a few labor-associated genes in the amnion. Gene activating histone-3 and -4 acetylation (H3ac, H4ac) and histone-3, lysine-4 demethylation (H3K4me2) have been reported at the promoter of the *PTGS2* gene, which encodes a key enzyme of prostaglandin biosynthesis (Mitchell et al., 2008, 2011). Further, H4ac levels were significantly elevated at term labor when *PTGS2* expression increased (Mitchell et al., 2008). Histone-3, lysine-4 trimethylation (H3K4me3) and histone-3, lysine-27 trimethylation (H3K27me3), which are activating and repressive chromatin marks, respectively, were assessed at the promoters of *PTGS2* and two other inflammatory genes, *NAMPT/PBEF*/visfatin and *BMP2*, in amnion tissues collected in early pregnancy (10–18 weeks) and at term (Mitchell et al., 2019). The expression of these genes increased robustly at the end of gestation. Both histone modifications were present at the promoters, and sequential double ChIP showed that the same promoter copies were marked by H3K4me3 as well as H3K27me3, indicating epigenetic "bivalence." Remarkably, bivalence was significantly reduced at term by the loss of the repressive H3K27me3 mark, indicating a shift toward a state poised for expression. H3K4 methyl transferases and H3K27me3 demethylases were expressed increasingly in the tissues with advancing pregnancy, potentially mediating the changes in histone methylation. This study suggests that an epigenetic process activating bivalently marked genes participates in the mechanism stimulating labor at term. The concept, however, has to be corroborated by the genome-wide profiling of H3K4me3 and H3K27me3 levels at gene regulatory regions in fetal membrane cells collected at different times during gestation.

Histone deacetylases are a diverse group of chromatin-modifying enzymes comprising 18 members classified into four groups (Seto and Yoshida, 2014). They are excellent drug targets, and histone deacetylase inhibitors and activators of varying isoform selectivity can be used as tools to explore the involvement of histone acetylation in gene expression control. Using this approach, Poljak et al. (2014) determined that Class II histone deacetylases may participate in the up-regulation of matrix metalloprotease-9 (MMP9) expression by ILβ in cultured amnion cells, while Class III histone deacetylases inhibit it. Similarly, the histone deacetylase inhibitor, TSA (Trichostatin A), reduced ILβ-stimulated PTGS2 expression in amnion explants supporting a role of histone acetylation in the action of the cytokine (Mitchell, 2006). The involvement of histone acetylation in these activities still needs to be confirmed by demonstrating cognate operational changes in acetyl histone levels at the gene regulatory regions since many non-histone proteins are also acetylated and are substrates for histone deacetylases (Seto and Yoshida, 2014). Therefore, drugs interfering with protein acetylation may cause global changes in the acetyl-proteome of the cells with functional consequences not necessarily mediated by histone acetylation-dependent epigenetic events (Norris et al., 2009; Orren and Machwe, 2019). An example for this has been found in the pregnant human myometrium, where TSA treatment *ex vivo* preserves progesterone receptor expression in its non-laboring state (Ilicic et al., 2017) potentially by epigenetic mechanisms (Ke et al., 2016; Ilicic et al., 2019) and reduces contractility by an extranuclear action that increases heat shock protein 20 acetylation promoting actin depolymerization and relaxation (Karolczak-Bayatti et al., 2011).

Non-coding RNAs

Transcription is not restricted to chromatin regions encoding protein-coding genes, but it is widespread throughout the genome. The resulting non-coding RNAs vary in size and function (Kung et al., 2013). The long non-coding RNA class, called lncRNAs, are over 200 bases long and have been implicated in epigenetic regulation by recruiting chromatin modifying protein complexes to the DNA regions to which they are tethered by complementary sequences. A well-characterized example is Xist RNA, which directs polycomb-regulatory complex-2 (PRC2) (catalyzing H3K27me3-dependent repression) to the X chromosome during X chromosome inactivation [reviewed by Kung et al. (2013)]. Long ncRNAs have been implicated in a range of reproductive disorders via epigenetic mechanisms (Shen and Zhong, 2015). Profiling of lncRNAs in (villous) placenta using a dedicated microarray covering over 33,000 (curated) lncRNAs (Arraystar Human LncRNA Array v2.0) revealed numerous differentially expressed lncRNAs in association with term and preterm birth and pPROM in two studies (Luo et al., 2013, 2015).

Ten differentially expressed natural antisense lncRNAs have been paired with differential mRNA expression from the same loci arguing for functional relationships (Luo et al., 2015). It will be important to extend these studies to the fetal membranes since integrated lncRNA, chromatin modification and gene expression profiling could reveal lncRNA-mediated epigenetic events involved in normal birth, pPROM and immune regulation in tissues covering most of the maternal-fetal interface.

A distinct group of lncRNAs, called pri-miRNAs, is processed into small, 22 nucleotides long RNA fragments, called micro-RNAs (miRNAs). Micro-RNAs act as guides to direct protein complexes to mRNAs or non-coding RNAs by sequence recognition (Hammond, 2015). Depending on the degree of complementarity, this results in (m)RNA degradation and/or the inhibition of mRNA translation to proteins. By conservative estimate, there are 2,300 human miRNAs validated to date (Alles et al., 2019). Because of the tolerant complementarity, a particular miRNA may be predicted to target numerous, possibly hundreds, of mRNA species or non-coding RNAs and a particular RNA species can concurrently interact with several miRNAs. Micro-RNAs, therefore, are proposed to have homeostatic roles providing robustness to cell phenotypes by dampening the effects of stochastic fluctuations of transcription (O'brien et al., 2018). Remarkably, the miR-200 family and the ZEB transcription factors were proposed to participate in a bi-stable double negative feedback loop that controls epithelial-mesenchymal transition in epithelial cell lines. The phenotype switch may be triggered by TGFβ and is reinforced by DNA methylation at the miR-200c∼141 promoters (Gregory et al., 2011). This interaction is just one example of the crosstalk between the micro-RNA and chromatin modification aspects of epigenetic regulation. Genes encoding micro-RNAs both at intergenic and intronic locations are subject to regulation by DNA methylation and histone modifications. The control is reciprocal, since DNMTs, TATs and histone modifying enzymes are targeted by micro-RNAs in normal and diseased (e.g., cancerous) cells (Chhabra, 2015; Yao et al., 2019). In the amnion, TGFβ-driven epithelial-mesenchymal transition occurs reversibly during gestation and apparently irreversibly at labor leading to membrane rupture (Janzen et al., 2017). Many molecular details of this process have been determined recently (Richardson et al., 2020); however, the contribution of epigenetic mechanisms, including miRNAs, is still unknown despite numerous possibilities identified in cancer cells undergoing analogous changes of phenotype (Serrano-Gomez et al., 2016). Epigenetic regulation underpinning the epithelial-mesenchymal transition of amnion cells is a promising new frontier of fetal membrane research.

Micro-RNAs in the Fetal Membranes

Montenegro et al. (2007) have profiled miRNAs in chorioamnionic membrane samples (with attached decidua) using the TaqMan MicroRNA qRT-PCR Assays Human Panel (Applied Biosystems–Early Access kit), which assays 157 miRNAs. Most (>150) of the tested miRNAs were detected, of which 13 had decreased levels with advancing pregnancy in women after preterm birth without histological chorioamnionitis. No differences were detected with term labor. In a subsequent study by the same group (Montenegro et al., 2009), 455 miRNAs were tested using the miRCURY LNA (Exiqon) microarray (v.8.1). Here, 39 differentially expressed miRNAs were found and most (79.5%) showed lower expression at term labor compared to preterm labor. One of them, miR-338, was verified experimentally in decidual cells to target *PLA2G4B* mRNA, which encodes a phospholipase involved in prostaglandin biosynthesis. They have also demonstrated a marked down-regulation of *Dicer*, a key enzyme of miRNA biogenesis, with advancing pregnancy in agreement with a widespread reduction of miRNA levels. This finding suggests that the homeostatic role of miRNAs, which is to stabilize a transcriptome that maintains the pregnancy-supporting phenotype of the membranes, is weakened at term, thus facilitating the transition to a labor-promoting state. An even higher number of miRNAs (875 miRNAs included in the miRCURY Array v.11 from Exiqon) have been tested in isolated amnion tissues at term and after preterm labor (Kim et al., 2011). This analysis found 32 differentially expressed miRNAs between the placental and extra-placental (reflected) regions of the amnion with 31 exhibiting lower levels in the reflected part. Moreover, down-regulation of the miR-143/miR-145 cluster has been verified by qRT-PCR in the reflected amnion at term labor and miR-143 has been shown to target *PTGS2* mRNA in a transfection assay with amnion mesenchymal cells. Collectively, the above series of studies suggests that a widespread decrease in miRNA expression plays a role in the labor-promoting proinflammatory switch in the fetal membranes at term. Post-transcriptional de-repression of genes of the prostaglandin biosynthetic pathway has been identified by targeted experiments as part of this process.

The miRNA profile of term amnion has been examined in obese women (with pre-pregnancy body-mas index >30) by Nardelli et al. (2014) using the TaqMan human MicroRNA Panel v.1.0, which contains 365 miRNAs. Seventy one percent of the tested miRNAs were detected in the amnions, of which 7 miRNAs were found only in obese women. The study also found 25 miRNAs that were differentially expressed in obese versus non-obese mothers. Further, Enquobahrie et al. (2015) explored the association of 8 preselected miRNAs with preterm birth of various clinical presentations (spontaneous, pPROM, pre-eclampsia) in the amnion and the chorion leave, based on the involvement of these miRNAs in placental pathologies (Enquobahrie et al., 2015). The two fetal membrane tissues expressed these miRNAs differentially, and miR-210 and miR-233 levels in the amnion, but not in the chorion leave, were inversely associated with preterm birth risk. These pioneering studies indicate that unfavorable conditions affect miRNA expression in the amnion, which may contribute to adverse pregnancy outcomes.

DECIDUA

The decidua is the endometrium of pregnancy and, being a maternal tissue, is not part of the *fetal* membranes by strict definition. It forms the maternal side of the maternal-fetal contact

zone, however, and is fused with the chorion leave so intimately that it is practically impossible to separate them completely, even with sharp dissection (Mitchell and Powell, 1984). The close contact predicts functional interactions, which warrants including an overview of the epigenetics of the human decidua in this chapter to provide context for the anatomical unit often referred to as the "choriodecidua." We focus on decidual stromal cells acknowledging that the decidua in pregnancy contains a complex and dynamic array of leukocytes (Gomez-Lopez et al., 2010), which are also subject to epigenetic regulation (Kim et al., 2012; Walsh et al., 2017). Comprehensive reviews, including the epigenetic aspects of decidual differentiation, have been published (Guo, 2012; Gao and Das, 2014; Liu et al., 2019).

DNA Methylation

Differentiation of the endometrium to decidua involves the transformation of endometrial stromal cells to decidual cells (Zhu et al., 2014), which has similarities to mesenchymal-to-epithelial phenotype transition (Zhang et al., 2013). It occurs in the non-pregnant uterus during the progesterone-dominated secretory phase of the menstrual cycle and its proper execution is essential for successful pregnancy. The role of DNA methylation in the process was explored initially by determining the expression of DNMTs in the endometrium during the menstrual cycle and in *in vitro* models, where decidual differentiation was induced by combined progestogen (progesterone, P4 or medroxyprogesterone acetate, MPA) and estradiol (E2) or cAMP treatments of cultured endometrial stromal cells (Yamagata et al., 2009; Vincent et al., 2011; Grimaldi et al., 2012; Logan et al., 2013). These studies showed down-regulation of DNA methyl transferase expression during decidualization. Likewise, 5-AZA (a DNA methyl transferase inhibitor) treatment fostered a phenotype in the endometrial culture system reminiscent of the decidual state (Logan et al., 2010). In spite of these observations, no change in the global level of DNA methylation has been detected (Grimaldi et al., 2012), suggesting that DNA methylation changes during decidual transformation may involve alterations of methylation pattern rather than changing the overall methylation degree. CpG site-specific methylation during decidualization *in vivo* has been investigated using the Illumina HM27k array (Houshdaran et al., 2014). The top 10% of probes reporting variable CpG methylation (2,578 probes) effectively separated samples from the proliferative and secretory phases of the cycle by unsupervised cluster analysis. Differential methylation analysis, however, identified just 66 CpGs with altered methylation, with several of them associated with genes important in endometrial biology. The follow-up study using primary endometrial fibroblasts decidualized *in vitro* by P4 and E2 indicated methylation changes at several CpGs and associated genes detected *in vivo* (Houshdaran et al., 2014). In a similar study, Maekawa et al. (2019) used the more comprehensive HM450k array and found only 23 differentially methylated CpGs after MPA + E2 treatment without change in the expression of the associated genes. Moreover, the DNA methylation status of the decidual marker genes *PRL* and *IGFBP1* showed no change after differentiation induced by MPA + E2, as determined by bisulfite (clonal) sequencing. Collectively, these data suggest that DNA methylation dynamics is slight in the decidua and the involvement of CpG methylation in the decidualization process is subtle. Other epigenetic processes, such as histone modifications, may play a more predominant role.

Histone Modifications

Decidual transformation of endometrial stromal cells *in vivo* and *in vitro* is associated with the decreasing expression of EZH2, which is the catalytic component of the histone methyltransferase complex, PRC2 (Grimaldi et al., 2011). PRC2 methylates the lysine-27 residue of histone-3, establishing the repressive H3K27me3 modification. Reduced EZH2 activity was accompanied by lowered H3K27me3 levels at the *PRL* and *IGFBP1* genes and the increased expression of the gene products, prolactin and insulin-like growth factor-binding protein 1, which are the best characterized markers of decidualization. However, the global H3K27me3 level remained unaltered after EZH2 down-regulation, indicating the locus selectivity of the histone modification changes (Grimaldi et al., 2011). Genome-wide survey of H3K27me3-marked sites by chromatin immunoprecipitation coupled to microarray promoter analysis (ChIP-chip technology, using the NimbleGen Human ChIP-chip 3 × 720 K RefSeq promoter array) confirmed the global rearrangement of the H3K27me3 pattern. Upon decidual transformation, H3K27-acetylation, which is the alternative modification of the H3K27 sites marking active gene promoters and enhancers, exhibited a marked global increase, including the *PRL* and *IGFBP1* genes, indicating widespread gene activation (Grimaldi et al., 2011). Subsequent studies in essence confirmed these findings by both genome-wide and candidate gene approaches (Tamura et al., 2014, 2018; Katoh et al., 2018) and implicated another gene-activating histone modification, H3K4me3, in the transformation process. Further, insulin signaling was one of the major pathways associated with genes up-regulated by the two activating histone modifications (Tamura et al., 2014). Following up on this line of investigations, Jozaki et al. (2019) determined that decidualization was dependent on the availability of glucose for the endometrial cells. In low glucose medium acetylation of the *FOXO1* promoter regions and expression of *PRL* and *IGFBP1* were suppressed. FOXO1 is a transcription factor with pivotal involvement in decidualization (Grinius et al., 2006; Park et al., 2016). Remarkably, glucose up-regulated *FOXO1* expression and promoter H3K27 acetylation in the decidualizing endometrial stromal cells, which in turn induced *PRL* and *IGFBP1* expression associated with promoter H3K27 acetylation (Jozaki et al., 2019). Although the histone acetyl transferase(s) that function in the glucose sensing mechanism were not identified, the results further support the fundamental role of epigenetic histone modifications in decidual transformation.

At term, the decidua acquires a pro-inflammatory phenotype (Norwitz et al., 2015) that involves the emergence of myofibroblast cells (Nancy et al., 2018). Nuclear accumulation of the H3K27me3 demethylase, lysine demethylase 6A (KDM6A), has been detected concomitantly with this process, which suggests that epigenetic mechanisms, including the removal of the suppressive H3K27me3 mark, take part in the transition

(Nancy et al., 2018). Very recently, a comprehensive analysis of chromatin landscape changes has been reported in endometrial fibroblasts decidualized *in vitro* (Sakabe et al., 2020). Chromatin accessibility using the ATAC-seq technique, ChIP-seq with H3K4me1, H3K27ac, and H3K4me3 antibodies, and promoter capture Hy-C to detect distant regulatory elements, were integrated and matched with RNA-seq data in this large-scale genome-wide study. In general agreement with previous findings, the results showed extensive changes during decidual transformation, particularly at enhancer sites marked with H3K4me1 and H3K27ac. Importantly, these chromatin data were also integrated with a genome-wide association study (GWAS) dataset that involved 43,568 women and explored genetic associations with gestational duration and preterm birth (Zhang et al., 2017). The computational integration of the GWAS with the functionally annotated chromatin regions in decidualized cells increased the hereditability estimates of gestational duration and resulted in the discovery of additional non-coding chromatin loci and associated genes, such as the gene encoding the transcription factor, Heart And Neural Crest Derivatives Expressed 2 (HAND2), potentially linked to gestational length (Marinić et al., 2020). From the epigenetic perspective, these studies have revealed that epigenetically controlled genomic loci involved in decidual transformation are critical for determining gestational length in women.

Non-coding RNAs

Information about miRNAs controlling endometrial receptivity and decidualization has been reviewed previously, highlighting the Let-7, miR-200, miR-181, miR-542, and miR-30 family and miR-17-92 cluster members in these events (Liu et al., 2016, 2019). Steady expression of Let-7 miRNA isoforms has also been reported in late gestation fetal membranes/decidua (Chan et al., 2013). In decidualization models of human endometrial stromal cells *in vitro*, two microarray analyses tested 435 (Qian et al., 2009) and 1,205 (Tochigi et al., 2017) human miRNAs for differential expression. In the former study, 16 and 33 miRNAs were found to be up- and down-regulated, respectively, while in the latter study, 1 was shown up-regulated and 5 down-regulated under similar threshold criteria. There was no overlap between the two differential expression datasets. Likewise, miR-181a up-regulation was found critical for decidual transformation in one study (Zhang et al., 2015), while miR-181, -183, and -200 family members were found down-regulated in decidualized cells other investigations (Qian et al., 2009; Estella et al., 2012) reporting that reduced hsa-miR-222 expression was particularly crucial (Qian et al., 2009). Jimenez et al. (2016) described the participation of up-regulated miR-200 in decidualization *in vitro*, and potentially *in vivo*, as part of the miR-200/ZEB regulatory network (Gregory et al., 2011), which confirms that endometrial stromal cells undergo mesenchymal-to-epithelial transition during decidual transformation (Zhang et al., 2013). Furthermore, *in vitro* decidualization also appear to involve the induction of the long intergenic non-coding RNA, LINC00473 (Liang et al., 2016). Further work shall reconcile the incongruous data from different laboratories and reveal a consistent picture about the participation of particular RNA species in decidual cell differentiation and function. The importance of short and long non-coding RNAs in these processes, however, is beyond doubt.

At term labor, down-regulation of microRNAs targeting inflammatory genes has been detected in (unseparated) choriodecidua samples leaving the contribution of the maternal (decidua) and the fetal (chorion laeve) components to be determined (Montenegro et al., 2009; Stephen et al., 2015).

Finally, there is considerable interest in defining the role of decidual non-coding-RNAs in the pathogenesis of early pregnancy loss (Dong et al., 2014; Wang et al., 2015; Hong et al., 2018; Zhao et al., 2018), pre-eclampsia (Zhao et al., 2014; Lv et al., 2018; Tong et al., 2018; Moradi et al., 2019) and in the establishment of the persistent decidual phenotype after implantation (Lv et al., 2016; Wang et al., 2016). Alterations of miRNA profiles have been demonstrated under these conditions, but the detailed discussion of these aspects of miRNA involvement are beyond the scope of this review.

DISCUSSION

The information currently available about epigenetic events in the fetal membranes and the decidua establishes structural changes of the chromatin as an important contributor to gene regulation in these tissues throughout pregnancy and in labor. The major types of chromatin modifications, such as DNA (CpG) methylation and histone modifications, have been found and many of them located in genome-wide and candidate gene-focused studies. Alterations in modification levels and patterns have been detected in association with gestational age, labor status and pathological conditions. Long and short non-coding RNAs (miRNAs), which represent a separate, but connected, branch of epigenetic regulation, have also been described and studied in detail. **Figure 1** illustrates the overall gestational dynamics of DNA methylation, histone modifications and micro-RNA expression in the amnion and the decidua as deduced from the currently available data. The periods around implantation, early pregnancy and preparation for birth are the most active for epigenetic events to occur. Environmental and disease-associated inputs can impinge upon the fetal membranes throughout pregnancy, but epigenetic mechanisms are most likely to be influenced at these dynamic periods. Acknowledging all the advances and the increasing pace of new discoveries it remains clear that fetal membrane epigenetics is still in an early stage of development. Some critical gaps of knowledge and foreseeable future directions are indicated in the preceding sections and several more are outlined below.

- Most data about epigenetic events in the fetal membranes describe associations between chromatin modifications, non-coding RNAs and outcomes related to various physiological or pathological states of pregnancy. Associations do not prove causation; therefore, inferences about epigenetic involvement remain tentative. Evidence supporting functional roles can be generated by interventional studies that target components of the epigenetic machinery using inhibitors of chromatin

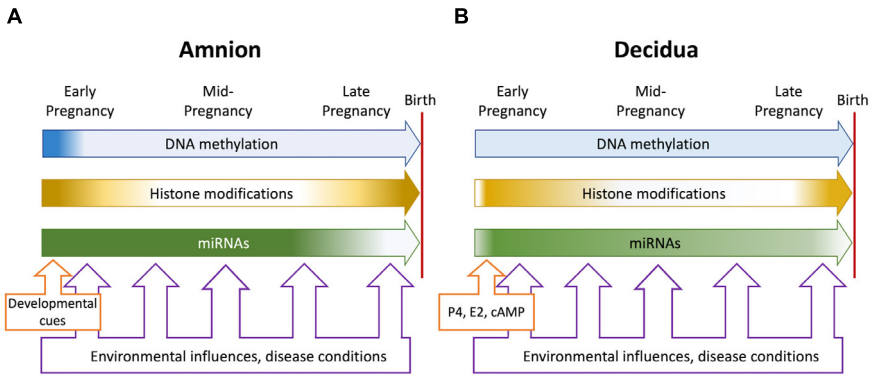

FIGURE 1 | Overall dynamics of epigenetic events in the amnion **(A)** and the decidua **(B)** during pregnancy. The blue, orange, and green block arrows indicate DNA methylation, histone modifications and miRNAs, respectively. Shading denotes changing levels and shifting genome-wide distributions. In the amnion, DNA methylation and histone modification patterns are established in early gestation to support pregnancy. Histone modification patterns change at term when labor-associated inflammatory genes are activated, and tissue remodeling occurs. Micro-RNAs stabilize the protective transcriptome until term, when levels decline concomitantly with inflammatory gene activation. In the decidua, hormonal influences (progesterone, estrogens, and cAMP signaling) trigger differentiation to the pregnancy-protective phenotype. The process involves major changes in histone modifications and miRNA expression but relatively modest alterations in DNA methylation. At term, histone modifications change, and key miRNAs decline to foster a proinflammatory and labor-promoting phenotype. Environmental adversities and disease conditions may be present throughout pregnancy but most likely impact on epigenetic events during periods of dynamic change. The resulting epigenetic "footprints" may influence gene expression patterns contributing to fetal membrane disfunction and may signify fetal exposure to unfavorable intrauterine conditions.

modifying enzymes and specially designed epigenetic chemical probes (Wu et al., 2019). A few of these studies have been published with fetal membrane cells or tissues so far (Mitchell, 2006; Logan et al., 2010; Poljak et al., 2014) and the results highlight the need for careful consideration of off-target effects and toxicity. Other options including the genetic manipulation of chromatin modifying enzyme and/or epigenetic reader levels and activity still need to be exploited in fetal membrane research. Correlations with polymorphisms of epigenetic effector genes may provide corroborating, but still associative, evidence for epigenetic involvement in fetal membrane regulation. Polymorphisms of the DNMT3B and DNMT3L genes were found to associate with familial preterm birth and birth weight, respectively, but fetal membrane involvement (e.g., associations with pPROM) was not reported (Haggarty et al., 2013; Barisic et al., 2020).

- Fetal membrane tissues *in vivo* are subject to environmental exposures and lifestyle conditions such as smoking, diet, toxic substances and effects of social stress. Epigenetic mechanisms mediate the long term (even transgenerational) effects of these exposures and conditions as determined in animal models (Suter and Aagaard-Tillery, 2009). Furthermore, a subset of the epigenetic changes have been proposed to be of adaptive nature predicting future environmental challenges (Duncan et al., 2014). "Metastable epialleles," which are genomic loci that function as epigenetic sensors of the environment, are critical in the process. Metastable epigenetic loci have been found in animals (Rakyan et al., 2002), for example, the *agouti* locus in mice (Dolinoy et al., 2007) and were tentatively identified in humans (Waterland et al., 2010; Dominguez-Salas et al., 2014). Smoking, nutrition and vitamin C intake significantly alter the incidence of pPROM indicating their impacts on fetal membrane function (Siega-Riz et al., 2003; Kyrklund-Blomberg et al., 2005; Nabet et al., 2007; Myhre et al., 2013), but the epigenetic aspects of these effects remain to be explored.
- Epigenetic events in the chorion leave are much less studied than in the amnion and the decidua. The chorion leave constitutes the fetal side of the maternal-fetal contact zone and a role in establishing and maintaining the maternal tolerance of the fetus is implied by its location (Kim et al., 2015). Chorion leave trophoblasts have a phenotype distinct from villous trophoblasts (e.g., chorion leave trophoblasts do not form syncytia) and possess a unique DNA methylation pattern (Eckmann-Scholz et al., 2012; Robinson and Price, 2015). Understanding the epigenetic aspects of fetal membrane function will require the characterization of chorion leave cell chromatin structure on a level with the other fetal membrane components.
- The amnion, chorion and decidua contain several types of cells, and experimental results with tissue samples represent a sum generated by heterogeneous cell mixtures that often vary in proportions. Chromatin modification patterns are cell specific, and epigenetic differences predominant in a particular cell type may be masked by the average. Analysis of isolated individual cell types or correction for cellular heterogeneity using cell-specific markers are approaches that can alleviate the problem of variable cell composition and increase the robustness of results. Importantly, the expanding suite of single cell epigenomic techniques (Clark et al., 2016) offers exciting new possibilities to analyze the composition and phenotype dynamics of fetal membrane cell populations and will very likely become a major research direction in the future.

- To identify chromatin loci where epigenetic alterations occur during pregnancy, labor and pathological conditions will require the generation and bioinformatic integration of genome-wide DNA methylation and histone modification datasets, as these modifications buttress the chromatin together and function in a combined fashion (Roadmap Epigenomics Consortium et al., 2015). Inclusion of transcriptomic, chromatin accessibility (Buenrostro et al., 2015) and chromatin conformation capture (Kempfer and Pombo, 2020) data in the integrative analyses are expected to inform about the functional impact of chromatin modification changes. These approaches are technically complex and computationally demanding, but also extremely informative, as has been demonstrated recently with decidual cells (Sakabe et al., 2020).
- Lastly, it is important to emphasize that advanced, genome-wide approaches and the more traditional analysis of individual candidate genes supplement each other and pursuing them in combination can result in optimal outcomes. Validation of key findings in genome-wide screens, and mechanistic studies in culture systems in general benefit from focusing on genes and regulatory regions pinpointed by genomic scale analyses. Emerging unexplored epigenetic mechanisms like non-cytosine DNA methylation (Ji et al., 2018) and protein mediated inheritance (Harvey et al., 2018) are exciting future directions in fetal membrane research.

AUTHOR CONTRIBUTIONS

TZ conceptualized the work and drafted the manuscript. JP provided intellectual input, edited the manuscript drafts, and finalized the figure designs. Both authors contributed to the article and approved the submitted version.

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REVIEW
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The Role(s) of Eicosanoids and Exosomes in Human Parturition

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The roles that eicosanoids play during pregnancy and parturition are crucial to a successful outcome. A better understanding of the regulation of eicosanoid production and the roles played by the various end products during pregnancy and parturition has led to our view that accurate measurements of a panel of those end products has exciting potential as diagnostics and prognostics of preterm labor and delivery. Exosomes and their contents represent an exciting new area for research of movement of key biological factors circulating between tissues and organs akin to a parallel endocrine system but involving key intracellular mediators. Eicosanoids and enzymes regulating their biosynthesis and metabolism as well as regulatory microRNAs have been identified within exosomes. In this review, the regulation of eicosanoid production, abundance and actions during pregnancy will be explored. Additionally, the functional significance of placental exosomes will be discussed.

Keywords: exosomes, eicosanoids, prostaglandins, pregnancy, parturition, gestation, preterm labor

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INTRODUCTION

The fetal membranes perform unique functions to support fetal development and respond to signals for parturition. The correct timing for triggering this process is critical for the successful outcome of the pregnancy. The parturition process is mediated by a combination of signals from the fetus, placenta and mother. There are mainly two signallers of parturition that are interdependent and well reported, namely fetal endocrine signals and fetal growth-related signals (Challis et al., 2005; Menon, 2016; Mesiano, 2019). Both pathways directly and indirectly induce higher production of eicosanoids (particularly prostaglandins) which are important signaling molecules that affect the contractile activity of the myometrium leading to parturition (Challis et al., 2005; Reinl and England, 2015). Hence, administration of specific prostaglandins (E_2 or $F_{2\alpha}$) is proven to effectively induce labor and cervical ripening (E_2) in women. Additionally, a better understanding of the

Abbreviations: *5-LOX*, 5-lipoxygenase; *BLT1-2*, Leukotriene B4 receptor 1-2; *cAMP*, Cyclic AMP; *COX*, Cyclooxygenase; *CRH*, Corticotrophin-releasing hormone; *CysLT1-2*, Cysteinyl leukotriene receptor 1-2; *DP1-2*, Prostaglandin D_2 receptor 1-2; *EETs*, Epoxy-eicosatrienoic acids; *EP1-4*, Prostaglandin E_2 receptor 1-4; *ESCRT*, Endosomal Sorting Complexes for Transport; *EVs*, Extracellular vesicles; *FLAP*, Five-lipoxygenase activating protein; *FP*, Prostaglandin $F_{2\alpha}$ receptor; *GRO*α, Growth-related oncogene-α; *HETEs*, Hydroxy-eicosatetraenoic acids; *HPETE*, Hydroperoxyl eicosatetraenoic acid; *IL*, Interleukin; *ILVs*, Intraluminal vesicles; *IP*, Prostaglandin I_2 receptor; LTA_4, Leukotriene A_4; LTB_4, Leukotriene B_4; LTC_4, Leukotriene C_4; LTD_4, Leukotriene D_4; *LTs*, Leukotrienes; *MRPs*, Multidrug-resistance proteins; *MTOC*, Microtubule organization center; *MVB*, Multivesicular Bodies; *PG*, Prostaglandin; PGD_2, Prostaglandin D_2; *PGDH*, prostaglandin dehydrogenase; PGE_2, Prostaglandin E_2; $PGF_{2\alpha}$, Prostaglandin $F_{2\alpha}$; PGH_2, Prostaglandin H_2; *PGHS = PTGS-2*, prostaglandin endoperoxide synthase-2; PGI_2, Prostaglandin I_2; *TNF*α, Tumor necrosis factor-α; *TP*, Thromboxane receptor; TXA_2, Thromboxane A_2.

regulation of eicosanoid production and the roles played by the various end products during pregnancy and parturition has led to our view that accurate measurements of a panel of those end products has exciting potential as diagnostics and prognostics of preterm labor and delivery (Mitchell et al., 2015).

In this review, we explore the roles and distribution of eicosanoids in the human uterus and fetal membrane during parturition. We also describe exosome abundance during pregnancy and parturition. Finally, we discuss the potentially pivotal role of exosomes in distributing eicosanoids and the related diagnostic and therapeutic potential that this brings.

EICOSANOIDS

The term "eicosanoid" has evolved overtime as a definitive term for products of a family of polyunsaturated (C_{20}) fatty acids; including, but not limited to, lipoxins, leukotrienes, thromboxanes and prostaglandins. The biosynthesis of eicosanoids and their structural properties are well characterized in mammals (Smith, 1989). Eicosanoids are not stored, and their biosynthesis occurs in all mammalian tissues as a response to hormonal stimulation or mechanical trauma, acting as paracrine or autocrine modulators (Esser-von Bieren, 2017; Strauss and FitzGerald, 2019). Their actions are mediated by the activation of membrane receptors (Kim and Luster, 2007).

Eicosanoid Biosynthesis

A first essential and usually rate limiting step in eicosanoid biosynthesis is release of polyunsaturated (C_{20}) fatty acids from membrane phospholipid stores (Fitzpatrick and Soberman, 2001). Arachidonic acid is the major common precursor of eicosanoids and its release is precisely regulated by several types of phospholipase A_2 (Burke and Dennis, 2009) or phospholipase C and subsequent mono- and diacylglycerol lipases (Kano et al., 2009; Hanna and Hafez, 2018). Once released arachidonic acid is converted enzymatically to various eicosanoids via three main pathways (**Figure 1**): namely, the cyclooxygenase pathway, the lipoxygenase pathway, and the cytochrome P-450 epoxygenase pathway (Strauss and FitzGerald, 2019).

The major products of the cyclooxygenase (COX) pathway are prostanoids such as prostaglandins, thromboxanes and prostacyclin. There are two major COX enzymes that initiate the synthesis of prostaglandins, COX-1 is mainly expressed constitutively, and COX-2 is often induced via cytokines, growth factors and hormones (Herschman, 1996; Smith et al., 1996). Prostaglandin H_2 (PGH_2), a direct product of arachidonic acid release and reaction to COX enzymes, is converted to individual prostanoids that are tissue specific by the action of corresponding isomerases and synthases (Smith et al., 2000). For instance, thromboxane A_2 (TXA_2) synthase is expressed in platelets and macrophages; prostaglandin I_2 (PGI_2), also known as prostacyclin, synthase is expressed in endothelial cells and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) synthase is abundant in the uterus (Ni et al., 2003; Ueno et al., 2005).

The lipoxygenase pathway produces leukotrienes (LTs). Products of this pathway in leukocytes are part of the LT family of lipid mediators, whose synthesis is mainly initiated by inflammatory cells. Formation of LTs is initiated via hydroperoxyl eicosatetraenoic acid (HPETE) formation from arachidonic acid by 5-lipoxygenase (5-LOX). 5-LOX in turn requires the cooperation of an accessory protein known as five-lipoxygenase activating protein (FLAP). Most HPETE molecules are converted to leukotriene A4 (LTA_4). LTA_4 can serve *in vitro* as a precursor for the transcellular biosynthesis of lipoxins and can undergo multiple routes of transformation (Bäck et al., 2011).

The cytochrome P450 epoxygenase pathway produces mainly epoxy-eicosatrienoic acids (EETs) via the catalysis of monooxygenation of arachidonic acid (Smith, 1989; Strauss and FitzGerald, 2019). However, hydroxygenases can also convert arachidonic acid to hydroxy-eicosatetraenoic acids (HETEs) (Strauss and FitzGerald, 2019).

Transport and Function

Despite the lipid nature of eicosanoids, they do not penetrate the cell membrane freely. Efflux transporters, such as multidrug-resistance proteins (MRPs), are necessary to transport newly synthesized eicosanoids outside the manufacturing cells. Additionally, the cellular uptake of eicosanoids is regulated by organic anion transporter proteins (Funk, 2001). The abundance of eicosanoid receptors and transporters is a limiting factor for their action. Therefore, they are believed to act as local or paracrine effectors initiating specific biochemical reactions in certain tissues.

Due to the different mechanisms that eicosanoids can induce on the cellular level, there are discrete receptor for each compound that mediate its action within the cell (Olson and Ammann, 2007). Thus far, there are 13 distinct cloned and characterized receptors for eicosanoids, including nine for cyclooxygenase-derived prostanoids and four for lipoxygenase-derived leukotrienes (Funk, 2001; Narumiya and Furuyashiki, 2011; Woodward et al., 2011). The nine prostanoid receptors mediate eicosanoid actions via cyclic AMP (cAMP), phosphatidylinositol turnover and Ca^{2+} shifts (**Table 1**).

Despite the short lifespan of eicosanoids, their biological effects are robust. Their biological properties have been studied in many contexts such as the cardiovascular system, immune system, nervous system and gastrointestinal tract as well as in inflammatory settings (Strauss and FitzGerald, 2019). The roles of eicosanoids in reproductive physiology are extensively studied in seminal fluid (Samuelsson, 1963; Alexandre et al., 2007; Remes Lenicov et al., 2012; Szczykutowicz et al., 2019), luteolytic actions (Vijayakumar and Walters, 1983; Bennegård et al., 1991; Miceli et al., 2001) and uterine physiology in pregnancy (Peiris et al., 2017); however, the roles of specific eicosanoids are still being elucidated. In the following sections, we will focus on the role of eicosanoids in uterine physiology during pregnancy and parturition.

Eicosanoids in Pregnancy and Parturition

The strong relationship between eicosanoids and pregnancy has been recognized for many years. Eicosanoids have various roles

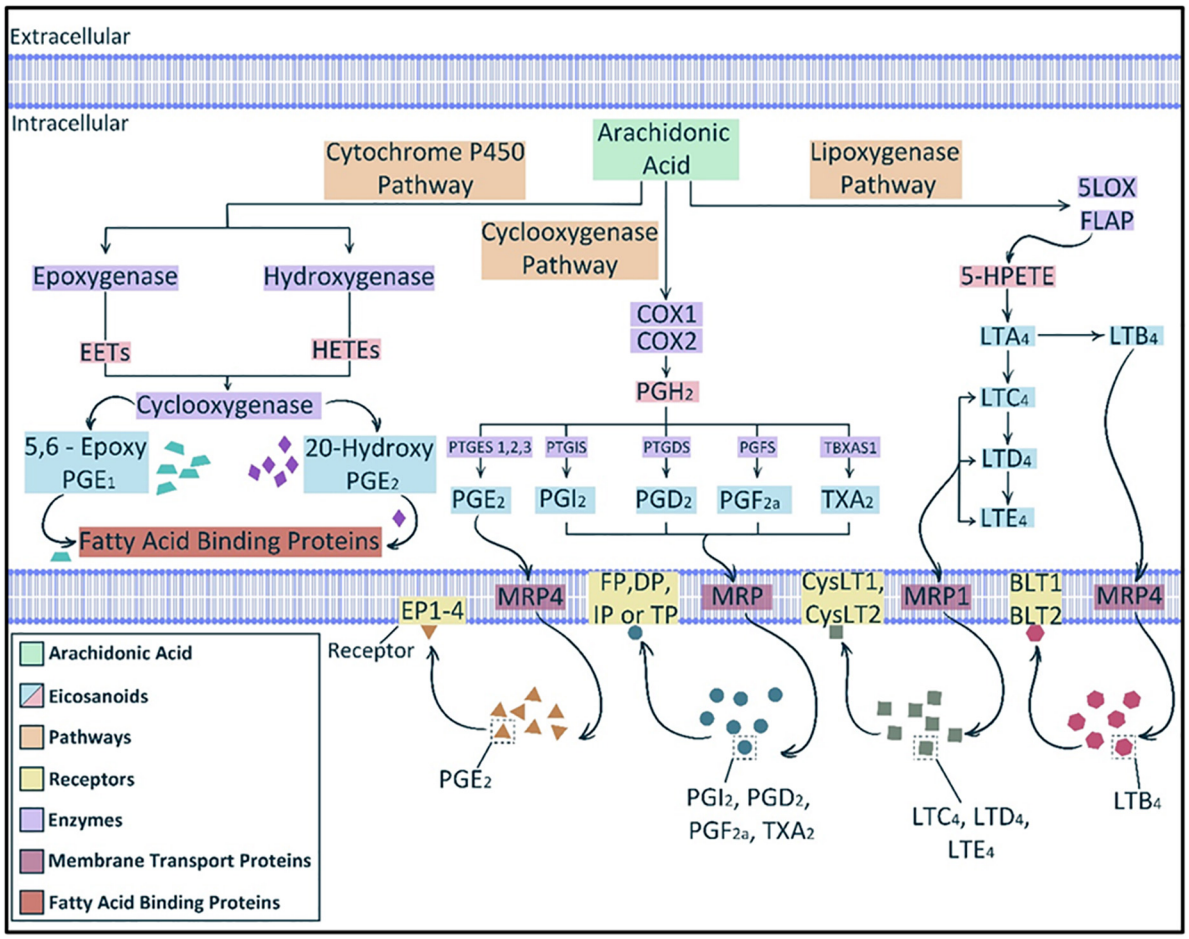

FIGURE 1 | Eicosanoid biosynthesis. The main three pathways involved in eicosanoids biosynthesis from the main precursor, arachidonic acid, are lipoxygenase, cyclooxygenase and cytochrome P450 pathways. The cytochrome P450 pathway produces epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs) from which some products can be further metabolized by cyclooxygenase. Cyclooxygenases (COX1 and 2) can act directly on arachidonic acid to produce the unstable intermediate prostaglandin (PG) H_2 (PGH_2) which then can produce various prostanoids such as PGE_2, PGI_2, PGD_2, and $PGF_{2\alpha}$ and thromboxane A_2 (TXA_2). Lipoxygenase pathway yields leukotrienes (LTs), such as LTA_4 and LTB_4. Multidrug resistant proteins (MRPs) facilitate the transfer of eicosanoids through the cell membrane. Multiple cellular membrane receptors mediate the action of eicosanoids, such as EP1-4 for PGE_2 and BLT1-2 for LTB_4. [After (Funk, 2001; Strauss and FitzGerald, 2019)].

TABLE 1 | Eicosanoid receptors and their functional properties.

Eicosanoid category	Ligand	Receptor	Functional properties
Cyclooxygenase pathway (prostanoids)	TXA_2	TP	Increase intracellular calcium, Contractile
	PGI_2	IP	Increase intracellular cAMP, Relaxing
	PGFzoc	FP	Increase intracellular calcium, Contractile
	PGD_2	DPI	Increase intracellular cAMP, Relaxing
		DP2	Induce intracellular calcium mobilization and chemoattractant
	PGE_2	EP1	Increase intracellular calcium, Contractile
		EP3	Inhibit cAMP production, Inhibitory
			Increase intracellular cAMP, Relaxing
Lipoxygenase pathway (leukotrienes)	LTB_4	BLT1 BLT2	Induce intracellular calcium mobilization and inhibit cAMP production
	LTD_4	CysLTl	Increase intracellular calcium
	LTC_4, LTD_4	CysLT2	Increase intracellular calcium

BLT1 -2: Leukotriene B_4 receptor 1-2; cAMP: Cyclic AMP; CysLT1-2: Cysteinyl leukotriene receptor 1-2; DP1-2: Prostaglandin D_2 receptor 1-2; EP1-4: Prostaglandin E_2 receptor 1-4; FP: Prostaglandin $F_{2\alpha}$ receptor; IP: Prostaglandin I_2 receptor; LTB_4: Leukotriene B_4; LTC_4: Leukotriene C_4; LTD_4: Leukotriene D_4; PGD_2: Prostaglandin D_2; PGE_2: Prostaglandin E_2; $PGF_{2\alpha}$: Prostaglandin $F_{2\alpha}$; PGI_2: Prostaglandin I_2; TP: Thromboxane receptor; TXA_2: Thromboxane A_2.

in the reproduction process, including ovulation, corpus luteum function, luteolysis, fertilization and decidualisation as well as parturition as previously reviewed (Strauss and FitzGerald, 2019). COX-2-derived PGE_2 was found to play an important role in oocyte maturation and fertilization by affecting the activity of the cumulus cells surrounding the oocyte (McAdam et al., 1999). Defective embryo implantation and decidualisation were also observed in COX-2-deficient mice uteri, indicating the fundamental role of PGs in normal uterus physiology (Lim et al., 1999; McAdam et al., 1999).

The importance of eicosanoids in parturition has been subjected to detailed investigations using knockdown animal models. For example, parturition defects were observed in rodents deficient of COX enzymes and $PGF_{2\alpha}$ receptor. Mice with targeted disruption of COX-1 gene had delayed parturition, resulting in neonatal death (Gross et al., 1998; Yu et al., 2005). $PGF_{2\alpha}$ receptor-deficient mice, generated by gene knockdown, did not show the normal decline of serum levels of progesterone associated with parturition and consequentially were unable to deliver normal fetuses at term (Sugimoto et al., 1997). Additionally, many clinical observations have accumulated evidences that demonstrate the likely regulatory function of PGs on myometrial contractility and cervical softening. For instance, administration of PGs biosynthesis inhibitors such as aspirin or specific COX-2 (also known as prostaglandin endoperoxide synthase-2; PGHS or PTGS-2) inhibitors extend gestational length, however, does not prevent parturition (Lewis and Schulman, 1973; Collins and Turner, 1975; Khanprakob et al., 2012; Illanes et al., 2014; Triggs et al., 2020). Likewise, administration of PGE_2 and $PGF_{2\alpha}$ at any stage of gestation leads to increasing uterine contractile activity and cervical ripening (Embrey, 1971). Consequently, PGs are used clinically as a treatment to induce labor (Thomas et al., 2014). Furthermore, production of PGE_2 and $PGF_{2\alpha}$ increases during late stages of gestation and were found to be associated with the onset of parturition (Romero et al., 1994a, 1996; Slater et al., 1999). This confirms the notion that increased intrauterine PG biosynthesis is a cause rather than a result of the parturition process.

Term Labor and Intrauterine Prostaglandin Concentrations

During pregnancy, there are two main groups of regulatory factors that control the contractile activity of the uterus, uterotropins and uterotonins. Uterotropins and relaxatory uterotonins, such as progesterone and PGI, respectively, enhance myometrial relaxation and modulate uterine function and growth (Ilicic et al., 2020). Contrarily, stimulatory uterotonins, such as PGs, can induce contractions of the uteri. Before the parturition process starts, a relaxation state of the myometrium with minimum sensitivity to stimulatory uterotonins, such as PGs, is controlled by progesterone (Mesiano, 2004; Ilicic et al., 2020). Progesterone is a key player in the establishment and maintenance of pregnancy and its role and regulation have been extensively studied in human and experimental models (Arck et al., 2007; Forde et al., 2009; Solano and Arck, 2020). Progesterone withdrawal usually indicates the initiation of the parturition process with changes in the contractile activity of the myometrium. Human parturition is also associated with progesterone receptor subtypes changes (Merlino et al., 2007; Patel et al., 2015).

Although the required enzymes and receptors necessary for the synthesis and action of PGs are present in human myometrial tissue (Astle et al., 2007; Arulkumaran et al., 2012), their concentrations in the uterus may vary during various stages of gestation. During pregnancy, both maternal and fetal tissues produce PGE_2 and $PGF_{2\alpha}$. The increased intrauterine prostaglandin concentrations are key players in initiating and progressing labor and this occurs before the onset of labor (Romero et al., 1994a, 1996).

During the initial stage of the parturition process, myometrial cellular expression of PG-related genes is significantly increased: these genes include PG biosynthetic enzymes and PG receptors (Challis et al., 2002). The changed expression of these genes in turn increases the uterine tissue sensitivity to the elevated production of PGE_2 and $PGF_{2\alpha}$. This leads to greater contractile activity that leads to expulsion of the fetus and sequentially expulsion of the placenta (Challis, 2013).

The balance between PG biosynthesis and metabolizing activities in the fetal membranes plays an important role in the parturition process. Intrauterine PG biosynthesis via PGHS occurs in the amnion and to a lesser extent in the chorion, decidua and myometrium. Conversely, prostaglandin dehydrogenase (PGDH) enzyme, which controls the conversion of PGE_2 and $PGF_{2\alpha}$ to their inactive forms, is predominantly expressed in the chorion before the onset of labor. This leads to the prevention of active amnion-derived PGs reaching the myometrium due to the abundant presence of PGDH in the chorion which lies between the amnion and maternal tissues (Mesiano, 2019).

During parturition, expression of PGHS increases in the chorion, decidua and myometrium. In the meantime, expression of PGDH decreases in the chorion. This leads to greater abundance of active PGs in the chorion and permitting more PGE_2 and $PGF_{2\alpha}$ to reach and induce their contractile action on the myometrium leading to progression of labor.

Of note, progesterone stimulates PGDH and has been reported to inhibit PTGS2 in the relaxed state of the myometrium before the onset of the parturition process (Pomini et al., 2000; Patel et al., 2003). Conversely, placental cortisol and corticotrophin-releasing hormone (CRH) can stimulate PTGS2 and inhibit PGDH, causing increased access of active PGs to the myometrium (Olson and Ammann, 2007).

PG receptors also play a crucial role in regulating PG action during human parturition. Receptors for PGI_2, PGE_2, $PGF_{2\alpha}$ and thromboxane are expressed in the myometrium during pregnancy (Grigsby et al., 2006). $PGF_{2\alpha}$ receptor (FP) and thromboxane receptor (TP) enhance contractions by increasing the intracellular calcium (Ricciotti and FitzGerald, 2011). Both PGI_2 and PGE_2 have contrary contractile actions on the myometrium. PGI_2 receptor (IP) mediates elevated levels of cAMP which in turn leads to relaxation. However, PGI_2 has been found to play a role in increasing expression of contraction-associated proteins, such as PTGS2 and PG receptors. Interestingly, PGE_2 has four different receptors (EP1–4) with different physiologic actions. While contractile

activity increases when PGE_2 interacts with EP1 and EP3, relaxation of the tissue can be mediated by PGE_2 interaction with EP2 and EP4 (Kotani et al., 1995). Therefore, PGE_2 can cause myometrial contraction or relaxation dependent upon the expression of receptor in different stages of pregnancy and during parturition.

A large literature illustrates the involvement of PGs in the five physiological events of human parturition: fetal membrane rupture via stimulating matrix mettaloproteinase activity and cell apoptosis (McLaren et al., 2000; Keelan et al., 2001), cervical ripening and dilation (Fletcher et al., 1993; Keirse, 1993; Steetskamp et al., 2020), myometrial contractility (Olson and Ammann, 2007), placental separation and uterine involution (Leung et al., 1987). This indicates the importance of further understanding the role of eicosanoids play in prognosis of pregnancy outcomes and their potential role as a diagnostic biomarker for fetus abnormalities and pregnancy complications, such as preeclampsia, gestational diabetes and preterm labor (Dalle Vedove et al., 2016; Hong et al., 2016; Aung et al., 2019; Welch et al., 2020).

Preterm Labor and Inflammatory Mechanisms

Labor that occurs before 37 completed weeks of gestation is considered as preterm, and preterm birth is the leading cause of perinatal mortality and morbidity (Goldenberg et al., 2008). The reasons behind the early onset of labor are not clearly identified (Green et al., 2005). Maternal infection is strongly correlated with preterm labor, such as intrauterine infection (Doi, 2020; Romero et al., 1994b). However, preterm delivery is associated with many other risk factors such as multifetal pregnancy, maternal obesity, maternal age, maternal nutrition and socioeconomic status (Johansson et al., 2014; Joseph et al., 2014; Koullali et al., 2016).

Inflammatory mechanisms are significantly involved in term and preterm labor (Christiaens et al., 2008; Peiris et al., 2019, 2020). Many studies focused on identifying labor-associated inflammatory genes profile, such as genes regulating cytokines, chemokines and related factors [reviewed in (Keelan et al., 2003)] which found to be upregulated in term deliveries and more apparently in preterm deliveries (Marvin et al., 2002; Mitchell, 2016). In term labor, infiltration of inflammatory cells increases in the cervix, myometrium, chorioamniotic membranes, and amniotic cavity. This is also found to be associated with increased expression and production of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNFα), and chemokines, such as IL-8 and growth-related oncogene-α (GROα) (Keelan et al., 2003; Romero et al., 2006). Cytokine regulation of intrauterine prostaglandin production was found to be at the biosynthesis level and the catabolic inactivation level. For instance, IL-1β and TNF-α enhance upregulated expression of PGHS leading to increased biosynthesis of prostaglandins by gestational tissues (Hansen et al., 1999; Rauk and Chiao, 2000). Similarly, pro-inflammatory cytokines, IL-1β and TNFα may inhibit PGDH leading to decreased degradation of prostaglandins (Brown et al., 1998; Mitchell et al., 2000). The role of pro-inflammatory cytokines in regulating prostaglandin production is further evidence of the importance of inflammatory mechanisms in mediating parturition (Gross et al., 2000; Peiris et al., 2019, 2020).

Similarly, infection and non-infection-induced inflammation have been found to be associated with preterm labor (Yoon et al., 2001; Romero et al., 2014, 2015). There are many experimental and clinical evidences in support of the involvement of inflammation in preterm labor. For example, pregnant animal models with intrauterine infection or with exposure to microbial products can lead to preterm delivery [reviewed in (Elovitz and Mrinalini, 2004)]. Extrauterine and sub-clinical intrauterine maternal infections have been associated with premature parturition (Gomez et al., 1995; Romero et al., 2006). The production of pro-inflammatory cytokines such as IL-1β, IL-8, and IL-6 are usually increased in the amnion, decidua and myometrium in pregnancies with infection (Goldenberg et al., 2000). This confirms the notion that parturition is a consequence of failure of the maternal immune system to regulate inflammatory mechanisms (Romero et al., 2006).

Eicosanoid Distribution and Measurement

Due to the importance of eicosanoids in pregnancy and parturition, the accurate and specific measurement of eicosanoids is critical to our ability to enhance diagnostic and therapeutic strategies for preterm labor. However, the misidentification of PGs has been problematic with traditional methodologies such as immunoassays (Glass et al., 2005). Previously we reviewed the molecular resemblance between eicosanoids and associated compounds that may interfere and affect the specificity of immunoassays (Glass et al., 2005; Peiris et al., 2017). Thus, the gold standard of mass spectrometry that allows full identification of PGs is vital to any meaningful approach to this problem (Peiris et al., 2020).

Prostaglandins are produced by all tissues in the body. Hence measurements of circulating concentrations reflect overall changes in production and cannot be directly linked to a specific tissue or organ source. Moreover, due to rapid clearance of circulating eicosanoids by lungs and kidneys (Golub et al., 1975; Dunn and Hood, 1977; Peiris et al., 2017), we can only assess circulating metabolites of eicosanoids not the original compounds. Therefore, there is a strong argument for evaluating the utility of exosomes (which have a content that reflects the tissue/cellular source) as a stable biomarker for measuring and identifying eicosanoids from specific organs such as the uterus.

EXOSOMES

Exosome Morphology

Exosomes are a subtype of membrane bound extracellular vesicles (EVs); they are 30–120 nm in diameter and have a cup-shaped structure and a lipid bilayer which is similar in orientation of transmembrane constituents to that of the parental cells membrane (Record, 2014; Barile and Vassalli, 2017; Shao et al., 2018). Exosomes contain a diverse array of biologically active molecules such as proteins, lipids, RNA (mRNA, microRNA and noncoding RNA), DNA, protein mediators and eicosanoids (Pillay et al., 2017; Saez et al., 2018). Exosomal contents comprise

specific proteins, lipids or genetic materials reflecting the source cell's physiological state, and can therefore serve as representative biomarkers (Menon et al., 2017).

Exosome Biogenesis

The biogenesis of exosomes involves the inward budding of the peripheral membrane and invagination of the late endosomes also known as Multivesicular Bodies (MVB) (Record, 2014); followed by the formation of intraluminal vesicles (ILVs) inside of the MVB's (Zhang et al., 2019). During the invagination process, proteins are incorporated into the membrane, leaving the cytosolic components to be engulfed into the ILVs (Zhang et al., 2019). MVBs are then fused with the plasma membrane of the cell releasing ILVs out into the extracellular space; once released ILVs are then referred to as exosomes (Kowal et al., 2014; Zhang et al., 2019; **Figure 2**). Exosomes biogenesis also requires; Endosomal Sorting Complexes for Transport (ESCRT) 0–III. This complex contains families of vacuolar sorting proteins, other associated proteins (e.g., Alix and tetraspanin) and lipids which also participate in protein sorting and ILV formation during biogenesis (Kowal et al., 2014). The selective packaging of molecules into exosomes occurs within the originating cell (Pillay et al., 2017); constituents within exosomes come from an array of cellular components such as the Golgi apparatus, endoplasmic reticulum, plasma membrane, nucleus and cytosol (Record, 2014), meaning that exosomes can represent many different parts of the cell. The few known selective mechanisms that regulate cargo sorting into exosomes have recently reviewed (Anand et al., 2019).

Exosome Secretion and Function

The exocytosis of exosomes is an active secretory process (Record, 2014). MVBs move along microtubules toward the cell's periphery fusing with the plasma membrane and causing the release of exosomes into the extracellular space (Zhang et al., 2019). Connection of the MVB and the microtubule organization center (MTOC) allows the sectorisation of exosome release, restricting the release of exosomes to non-random areas of the cell membrane (Record, 2014). Exosome release is also dependent on the cells and conditions of their surrounding environment (Kowal et al., 2014; Barile and Vassalli, 2017).

Once exosomes are released, they become involved in communication between cells through cargo delivery to the recipient cells. There are three main types signaling modes; autocrine affects the releasing cell, paracrine affects adjacent cells and endocrine is delivered to distal target cells via the circulation.

Exosomes are a device for both transportation and signaling; through their load of bioactive molecules, they have the innate ability to signal from inside a target cell; both from the periphery and intracellular compartments (Record, 2014).

The function of exosomes is to exchange information through the delivery of cargo to distal and adjacent target cells. In doing so, the interaction of target cells with exosomes results in reprogramming of their phenotype and regulation of their function; functions such as migration, proliferation, angiogenesis, translational activity, metabolism, and apoptosis (Ehrlich et al., 2016; Saez et al., 2018). This reprogramming and regulation consequently alters cellular physiology, and in some cases contributing to different pathological states (Pillay et al., 2017).

Exosomes in Pregnancy

Synthesis and Interactions With Surrounding Environment

Exosomes have been identified in the maternal circulation as early as 6 weeks into gestation (Salomon et al., 2014). As gestational age increases, there is an increase in circulating maternal exosome concentration (Pillay et al., 2017); with the increased exosome burden likely related to placental mass and derived primarily from placental mesenchymal stem cells.

First-trimester trophoblast cells act as environmental sensors, and these cells can respond to the changing environment via the synthesis and release of exosomes (Mitchell et al., 2015). For example, an increase of exosome numbers is observed when the *in vitro* environment has a low oxygen tension and is high in D-glucose concentration; these two factors synergistically interact to regulate the bioactivity and release of exosomes originating from first-trimester trophoblast cells.

The effect of environmental factors on the release of exosomes into the maternal circulation via endocrinal communication is dependent on the integrity and stability of exosomes (Ehrlich et al., 2016). For example, increased release of exosomes from trophoblastic cells is seen as a response to challenging environmental conditions (e.g., elevated glucose concentrations and low oxygen tension) which might disrupt the balance of cytokines (Truong et al., 2017). Cytokines being a necessity for healthy implantation, placentation and successful pregnancy outcome (Mitchell et al., 2015).

Exchange, Mediatory Roles, and Other Functions

A function of placenta-derived exosomes is to be a mediator in the progression of pregnancy and cell fate. Exosomes are used in cell-to-cell communication between the placenta and maternal organs. This communication has many functions, one of which is the preparatory function of remote tissues for metabolic and placental changes during gestation (Greening et al., 2016; Jin and Menon, 2018).

Basic functions of exosomes in normal uncomplicated pregnancies are promotion of implantation and communication between endometrium and embryo (Jin and Menon, 2018). *In vitro* studies have also revealed the role of exosomes in differential endothelial cell migration and vascular tube formation. Additionally, exosomes have a pivotal immunoregulatory role via the initiation of activated maternal lymphocytes' local deletion and induction of maternal t-cell apoptosis, which prevents the degradation of invading trophoblastic cells (Greening et al., 2016; Pillay et al., 2017). Placenta-derived exosomes are also found to play a role in viral infection during pregnancy, where trophoblast cells can transfer the necessary capacity of resistance against viral infection to other nonplacental cells via exosomes (Mouillet et al., 2014).

Exosomes regulate all these functions through the transference of their content into target cells. This regulation of activity

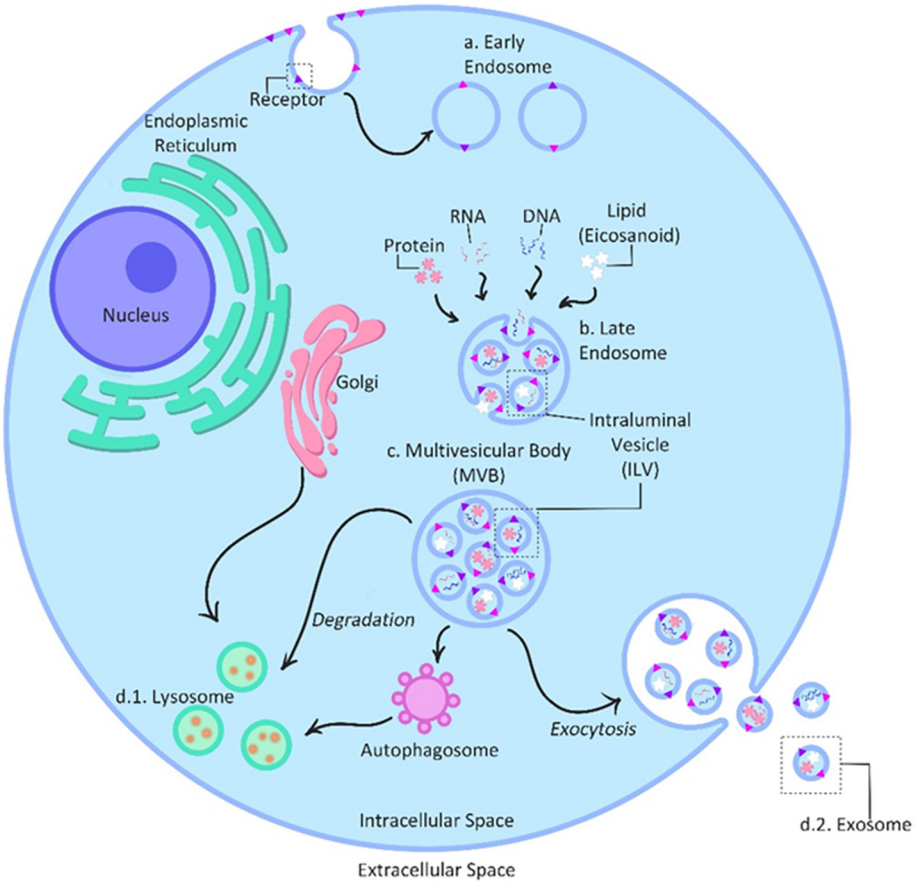

FIGURE 2 | Exosome biogenesis and secretion. The biogenesis of exosomes is initiated with the inward budding of the peripheral membrane into early endosomes **(a)**. Selective exosome packaging with molecules such as protein, RNA and DNA and lipids occurs within the originating cell and cellular compartments such as the Golgi apparatus, endoplasmic reticulum, plasma membrane, nucleus and cytosol. Invagination of the early endosomes into late endosomes **(b)** allows these diverse molecules to be taken up and individually packaged inside intraluminal vesicles (ILVs), turning the endosomes into multivesicular bodies (MVBs) **(c)**. During the invagination process, proteins are incorporated into the membrane, leaving the cytosolic components to be engulfed into the ILVs. Finally, MVBs either release ILVs intracellularly to be absorbed by lysosomes/autophagosomes **(d.1)** for degradation or fuse with the plasma membrane to secrete ILVs out into the extracellular space as exosomes **(d.2)**. [After (Kowal et al., 2014)].

can occur with either proximal or distal target cells via different interactions; this includes the modification of the extracellular milieu of the target cell, activation of cell membrane receptors, endocytosis by target cells in which the cell contents are released intracellularly and translational activity (e.g., angiogenesis, proliferation, metabolism and apoptosis). Exosomes can then modify the phenotype of these cells through maternal circulation.

The internalization of exosomes is also found to induce the release of pro-inflammatory cytokines (Greening et al., 2016). Exosomes released due to abnormal environmental factors lead to dysfunction of feto-placental endothelium and other various types of endothelial exosomes (Saez et al., 2018).

The involvement of exosomes in the transcellular metabolism of eicosanoids (and enzymes involved in substrate release for eicosanoids) has been described specifically and in terms of lipid mediators in a series of studies. In these studies exosomes from cells contained the full range of phospholipases and many free fatty acids (Subra et al., 2010; Record et al., 2014). The internalization of exosomes was described and the subsequent utilization of exosome cargo in cell metabolism (Subra et al., 2010) as well as involvement of this intercellular trafficking in pathophysiologies (Record et al., 2014). We have recently reviewed this in detail in an invited publication (Peiris et al., 2017).

Various studies have demonstrated the pivotal role of exosomes during human pregnancy and parturition (Sarker et al., 2014; Mitchell et al., 2015; Menon et al., 2017; Jin and Menon, 2018; Sheller-Miller and Menon, 2020). More interestingly, the potential role of exosomes in diagnosis/prognosis of pregnancy complications has gained a lot of attention in the scientific field in the last two decades. For example, preterm labor was one of the main topic that is under investigation (Cantonwine et al., 2016; Menon and Richardson, 2017; von Linsingen et al., 2017). Likewise, studies on gestational diabetes

(Powe, 2017; Liu et al., 2018; Saez et al., 2018) and preeclampsia (Pillay et al., 2016; Nielsen et al., 2017; Navajas et al., 2019) showed differential exosomal contents compared to that in uncomplicated pregnancies.

FINAL REMARKS

The intercellular communication mediated by exosomes has opened new era of research to study biological processes in healthy and pathophysiological conditions. From a clinical perspective, exosomes are mainly used in two applications: as biomarker detection and biologically active carriers. Exosomes are potential candidates as biomarkers detection tool circulating in blood. Enrichment of specific markers can be improved by exosome isolation and cargo identification (Record et al., 2018). Exosomes may transport proteins, lipids and nucleic acids that can be used as diagnostic or prognostic markers for specific clinical conditions. In this respect, several studies identified potential exosomal markers for early detection, diagnosis, and monitoring of cancer patients (Melo et al., 2015; Jalalian et al., 2019; Makler and Asghar, 2020). Similarly, exosomal contents are now gaining much attention in the field of pregnancy complications and fetal abnormalities (Yang et al., 2020). On the other hand, exosomes are now identified as potential platform for enhanced delivery of specific cargo in vivo, which can be biological compounds or therapeutic agents. The methods of loading exosomes with specific cargos of interest have been recently reviewed (Li et al., 2018; Donoso-Quezada et al., 2020; Mitchell et al., 2020). Exosomes have also inspired researchers to use cell-membrane-cloaked nanoparticles, also called synthetic exosome-mimics, as drug delivery platforms (Hu et al., 2011, 2015; Cao et al., 2016). These different applications of exosomes can provide hope to many patients including women with complicated pregnancies.

The relationship and importance of eicosanoids in pregnancy, labor and parturition are well established and have been an area of research for many decades. However, the limitations of immunoassays in the accurate measurement of specific eicosanoids have hampered research. The development of sensitive and specific mass spectrometry-based method to measure individual eicosanoids (e.g., prostaglandins and prostamides) via the monitoring of characteristic mass fragment pairs for each molecule at their distinct retention times has overcome these technical limitations and for the first time allowed accurate measurement of specific eicosanoids (Mitchell et al., 2016). A small but growing number of clinical studies have adopted mass spectrometric evaluations of these compounds, which has led to important new findings in the areas of labor and uterine infection (Maddipati et al., 2014, 2016; Peiris et al., 2020). The identification of the building blocks and enzymes needed for the synthesis of eicosanoids within exosomes is doubly exciting (Subra et al., 2010; Record et al., 2014). Firstly, the evaluation and quantitation of the cargo may provide a real-time snapshot of the cells' state. Secondly, exploration of the exosomes' abilities as vesicles of intercellular communication (i.e., to transport and deliver messages between cells) may further our understanding of the parturition process and provide opportunities to reconsider the mechanisms of pregnancy and parturition.

We postulate that together, understanding and quantitating eicosanoid biosynthesis, metabolism and actions in combination with exosome biology will enable the discovery of diagnostic and prognostic biomarkers for many pregnancy complications including preterm labor.

AUTHOR CONTRIBUTIONS

MM conceived the idea. EM and IMG prepared figures. EM, HP, OH, IMG, and MM contributed to manuscript writing. All authors contributed to the article and approved the submitted version.

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