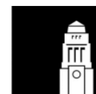

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1 **Leveraging Bioengineering to Assess Cellular Functions and Communication Within**

2 **Human Fetal Membranes**

3

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23

24 **Running head:** Mechanisms of fetal membrane paracrine signaling

25

26 **Keywords:** Organ-on-Chip, fetal membrane, immunology, preterm prelabor rupture of

27 membranes, preterm birth

28 **Abbreviations**: AEC, amnion epithelial cell; AMC, amniotic mesenchymal cells; CAM,

29 chorioamnionitis; COX, cyclooxygenase; CTB, cytotrophoblast; DAMP, damage-

30 associated molecular pattern; DSC, decidual stromal cells; E2, estrogen; ECM,

31 extracellular matrix; GM-CSF, granulocyte-macrophage colony stimulating factor;

32 IGFBP-1, insulin-like growth factor binding protein-1; Mφ, macrophage; NK, natural

33 killer; P4, progesterone; PAMP, pathogen-associated molecular pattern; PG,

34 prostaglandin; PDMS, polydimethylsiloxane; PPROM, preterm prelabor rupture of the

35 membranes; PRR, pattern recognition receptor; PTB, preterm birth

36

37 **Abstract**

38 The fetal membranes enclose the growing fetus and amniotic fluid. Preterm prelabor
39 rupture of fetal membranes is a leading cause of preterm birth. Fetal membranes are
40 composed of many different cell types, both structural and immune. These cells must
41 coordinate functions for tensile strength and membrane integrity to contain the growing
42 fetus and amniotic fluid. They must also balance immune responses to pathogens with
43 maintaining maternal-fetal tolerance. Perturbation of this equilibrium can lead to
44 preterm premature rupture of membranes without labor. In this review, we describe the
45 formation of the fetal membranes to orient the reader, discuss some of the common
46 forms of communication between the cell types of the fetal membranes, and delve into
47 the methods used to tease apart this paracrine signaling within the membranes,
48 including emerging technologies such as organ-on-chip models of membrane
49 immunobiology.

50 **Introduction**

51 The fetal membranes, also referred to as the extraplacental or gestational
52 membranes, extend from the placenta and form the wall of the sac that contains both
53 amniotic fluid and the developing fetus. This thin maternal-fetal interface serves both as
54 a passive, physical barrier and an active, immunological barrier to infection, while also
55 signaling to the developing fetus [1]. Bacterial infection or inflammation of the fetal
56 membranes (referred to as acute chorioamnionitis, CAM) can trigger preterm birth
57 (PTB), preterm prelabor rupture of membranes (PPROM), and can lead to stillbirth or
58 neonatal sepsis [2-4]. Unfortunately, CAM is often asymptomatic until a serious adverse
59 event occurs. There is a tremendous need to develop new approaches to prevent and/or
60 treat CAM *before complications occur*. However, such efforts are stymied by a lack of
61 robust tissue models of the fetal membranes that can be used to define how the host
62 responds to microbial threat *early* in the course of infection. Coordination between
63 multiple cell types within the membranes may be necessary for fetal membrane rupture.
64 The way these cells communicate between adjacent layers and coordinate function-
65 during gestation, term rupture, and preterm rupture- is not fully defined. In this
66 review, we will examine the development of the membranes, the most commonly
67 studied mechanisms of paracrine signaling within the fetal membranes, the model
68 systems used to discover these mechanisms, and the future of tissue modeling using
69 organ-on-chip technology.

70 Although technically comprised of maternal and fetal cells, this membrane is
71 commonly referred to as "the fetal membrane" and is comprised of several cell types
72 arranged in discrete layers. This is truly an allogeneic maternal-fetal interface. The non-
73 fetal portion of the membrane maternal-fetal interface is the maternal decidua, defined
74 by the abundance of decidual stromal cells (DSCs). Moving inwards is the fetal chorion,
75 primarily comprised of cytotrophoblasts (CTBs), then the amnion, which contains
76 mesenchymal cells (AMCs) and a thin layer of amnion epithelial cells (AECs) facing the
77 amniotic fluid and fetus (Figure 1). Immune cells including uterine natural killer cells
78 (uNK cells), macrophages (Mφ), and T and B lymphocytes are also present. NK cells are
79 found mostly in the decidua, the amnion and chorion are largely devoid of NK cells[5].
80 Likewise, the decidua appears to have greater numbers of immune cells relative to the
81 chorion and amnion[6].

82 *Formation of the fetal membranes*

83 Uterine stromal cells differentiate into DSCs during the progesterone phase of
84 the menstrual cycle in a process known as decidualization, and eventually become the
85 *decidua parietalis*, the maternal layer of the fetal membranes. The uterine lining must be
86 receptive to a fertilized egg and involves dynamic changes in gene expression: α-
87 smooth muscle actin is induced and the cells may resemble myofibroblasts [7]. Other
88 markers of decidualization include expression of vimentin (expressed in endometrial
89 stromal cells at lower abundance but dramatically upregulated during decidualization

90 [8]), desmin, prolactin, and insulin-like growth factor binding protein (IGFBP)-1 [7].
91 Decidualization can be induced *in vitro* using endometrial stromal cell lines or primary
92 endometrial stromal cells with progesterone (P4), estrogen (E_2), and cyclic AMP
93 (cAMP). Decidualization can be monitored by increases in secretion of prolactin and
94 IGFBP-1 over 6-12 days [7]. Decidualization induces expression of many functional
95 categories of genes; of note are genes involved in cell adhesion and cytoskeleton
96 organization [7]. Early microarray studies of DSCs differentiated from decidualized
97 fibroblasts showed broad changes in genes related to cell and tissue structure and
98 function (*e.g.* transporters, adhesion) and regulation of cellular processes (*e.g.*
99 inflammatory mediators, cell cycle)[7,9].

100 The capsular chorion frondosum, characterized by CTBs, forms villi, however
101 ultimately these villi and the associated intervillous spaces regress to fuse with the
102 apposed trophoblastic shell to give rise to the chorion leave [10]. The chorion laeve
103 grows with the fetus as a fibrous layer, and around 17 weeks post-conception fuses with
104 the parietal decidua on the opposite side of the uterus from where implantation
105 occurred.

106 The amnion is formed from amnion epithelial cells (AECs) and mesoderm which
107 is separated from the developing embryo by what will become the amnionic cavity
108 filled with fluid as the fetus enlarges. This single layer epithelium sits on a basement
109 membrane that is juxtaposed with the mesodermal connective tissue layer made up of a

110 compact stromal and fibroblast layer, which includes the amnion mesenchymal cells.
111 The amnion does not have any vascular supply and thus must be supported by the
112 surrounding amniotic fluid and blood vessels of the fetal surface of the placenta. The
113 mesoderms of the amnion and the chorion are ultimately closely apposed and only
114 separated by an intermediate spongy layer. Because of the spongy layer, they are never
115 truly fused and can be physically separated at term after birth.

116 In addition to the cell types making up the fetal membranes, there is a rich ECM
117 connecting and supporting the cell types. However, the ECM is not an inert scaffold.
118 The ECM harbors growth factors in its matrix[11]; integrin binding to ECM components
119 affects cellular signaling; and stretching of ECM results in release of cryptic matrikines,
120 which stimulate cytokine and matrix metalloproteinase (MMP) production[11]. The
121 ECM is actively maintained by cells, and stress hormones can reduce the production of
122 collagen[12]. MMPs, produced and secreted by immune and structural cells, break
123 down collagen - the products of which can stimulate further immune responses[13].
124 There are also tissue inhibitors of matrix metalloproteinases (TIMPs) acting to oppose
125 MMPs in the membranes[14]. Thus, the ECM plays a role in cell-to-cell communication
126 and should be considered when trying to recreate the conditions present in the fetal
127 membranes.

128 *Mechanical function of the fetal membranes*

129 One of the major roles of the fetal membranes is mechanical in that they must be
130 strong enough to encompass the growing fetus and amniotic fluid but also maintain
131 adequate elasticity for the same purpose. These properties are generally thought to
132 come from the collagen in the connective tissue components and extracellular matrix
133 (ECM) of the fetal membranes[15]. Although the thinner of the two membranes at just
134 50 μm, the amnion is stronger because of dense collagen. The chorion is four times as
135 thick (~200 μm) but contains collagen that is structurally less ordered. Imaging by
136 confocal microscopy identified the presence of collagen types I, III, IV, V, and VI in the
137 fetal membranes[16]. The decidua contains types I, III, IV, and V; the chorion contains
138 types IV and V; the reticular layer contains types I, III, IV, V, and VI, while the amnion
139 primarily contains types V and VI with type IV on the amnion epithelial surface[11].
140 The amnion and chorion are both highly hydrophobic, thought to decrease
141 intermembrane friction[17].

142 A discrete area of the fetal membranes in the supracervical region has decreased
143 strength compared to the rest of the membrane at late stages of pregnancy[18-20]. This
144 region also has increased expression of MMP9 along with decreased TIMP3, all
145 resulting in a so-called 'zone of weakness' with evidence of localized cellular
146 remodeling[21]. Supracervical membrane tissue demonstrated decreased release of
147 interleukin (IL)-1β, IL-6, IL-8, Tumor Necrosis Factor α (TNFα), and prostaglandin E_2
148 (PGE_2) with increase in signaling of transcription factor NF-κB at term before labor[22].

149 A putative mechanism for the changes of the supracervical membrane region involves a
150 dose-dependent decrease in membrane strength with increases in exposure to TNFα
151 and IL-1β[23] (both of which are increased at the end of pregnancy). This phenomenon
152 seems to be induced by collagen remodeling via changes in MMP9, TIMP3, and
153 Poly(ADP-Ribose) Polymerase (PARP)-1 cleavage. In women with PPROM, the chorion
154 was thinner and showed higher levels of apoptosis [24,25].

155 Some groups propose that the mechanical properties of fetal membrane stretch
156 may predispose the zone of weakness to enzymatic degradation of the surrounding
157 collagen [26]. Static mechanical stretch of fetal membrane explants increased
158 cyclooxygenase (COX)-2 and PGE_2 associated with NF-kB signaling [27]. *In vitro*
159 stretching of membranes causes overexpression of IL-8, interleukin-enhancer binding
160 factor 2, and huntingtin-interacting protein 2 [28].

161 *The fetal membranes during labor*

162 The fetal membranes during labor experience a myriad of changes to gene
163 expression, including a distinct upregulation of inflammatory genes [29,30], including
164 chemokines and their receptors, cytokines, matrix metalloproteinases, pattern
165 recognition receptors, and integrins. These changes are accompanied by the activation
166 of oxidative stress [31]. Not only does this tissue undergo changes in response to labor,
167 research suggests that fetal membrane aging, or senescence, may help trigger labor in
168 surrounding tissues [32-39]. Senescence refers to the cell age-associated telomere

169 shortening responsible for cell cycle arrest, and shortening of fetal membrane cell
170 telomeres was found by Menon *et al.* in 2012 [40]. This telomere shortening in the
171 membranes is linked to the p38 mitogen-activated signaling kinase pathway, leading to
172 a "senescence-associated secretory phenotype", which can signal to surrounding tissues
173 via secreted extracellular vesicles [41-43]. While many studies suggest that apoptosis in
174 the fetal membranes is a major causal event of weakening [23-25,44,45], this can be
175 confused for senescence in the membranes, which is more often accompanied by
176 inflammation [34,38,46].

177 **Communication within the fetal membranes: mechanisms of paracrine signaling**

178 The capacity for cells within the fetal membranes to orchestrate immune tolerance,
179 respond to infection and initiate labor signals requires a tightly-regulated and
180 integrated system of cell-to-cell signaling, which can be autocrine or paracrine. We now
181 turn in this review to provide an overview of key cell-cell signaling systems in the fetal
182 membranes.

183 *Pattern Recognition Receptors (PRRs)*: PRRs are expressed on many cell types, including
184 immune cells and structural cells of the fetal membranes, and they recognize conserved
185 motifs on pathogens and damaged host cells, known as pathogen-associated molecular
186 patterns (PAMPs) or damage-associated molecular patterns (DAMPs), respectively.
187 Major classes of PRRs include the toll-like receptors (TLRs), the Nod-like receptors

188 (NLRs) and the RIG-I receptors. PRRs stimulate the beginning of an immune response
189 through downstream signaling pathways resulting in antimicrobial or inflammatory
190 processes including cytokine production. Decidual cells are known to respond to
191 infection: they express multiple pattern recognition receptors including all TLRs at the
192 RNA level[47,48] and Nod-like receptors. Term decidual stromal cells have been shown
193 to express TLRs 1-6 at the protein level[49,50]. AECs and AMCs both express TLRs 2
194 and 4, although in AECs, TLR2 expression is limited to the basolateral side until an
195 inflammatory response is initiated, which then induces expression of TLR2 on both
196 sides [48,49,51,52]. AMCs also express TLRs 1-10 at the mRNA level and TLR6, in
197 addition to TLR2 and 4, at the protein level, and stimulation with TLR2/6 ligands
198 induces proinflammatory responses[53].

199

200 *Cytokines, chemokines, and growth factors in the fetal membranes:* Cytokines are proteins for
201 cell-to-cell communication that typically have a pro- or anti-inflammatory role, while
202 chemokines act as chemoattractants for subsets of leukocytes. The secretion of cytokines
203 and chemokines during inflammation helps to coordinate an immune response among
204 multiple cell types including monocytes, Mφ, lymphocytes, and structural cells of the
205 fetal membranes. There are several key players in the immune response to pathogens
206 during pregnancy which come up in the literature most often [30,54]. TNFα, among

other roles, contributes to membrane weakening [55] and activating MMP production [54]. Interleukin (IL)-6, which may alter the phenotype of recruited Mφ [56,57], is scarce in pre-labor membranes but is increased in the membranes following labor at term. Among other functions, IL-1α can induce nitric oxide synthesis in surrounding uterus [58] and β can alter the metabolism of prostaglandins involved in parturition [59]. Amnion mesenchymal cells stimulated with IL-1β were found to induce a sterile proinflammatory gene signature, including NFκB-related genes [60]. Finally, type 1 interferons (α and β) in turn can control chemokines such as CXCL10, among many other interferon-stimulated genes [61]. These cytokines have the potential to be produced by many structural and immune cells of the fetal membranes, but are tightly controlled, presumably to prevent uncontrolled inflammation in the membranes[23,54]. IL-8 (CXCL8, which is often induced by IL-1β [62] and is usually regarded as a neutrophil chemoattractant [63]), CCL2 (monocyte chemotactic protein, MCP-1, primarily associated with monocyte trafficking), and CCL3 (MIP-1α, Mφ inflammatory protein alpha, which induces secretion of cytokines and prostaglandins [61]) and CCL5 (RANTES) (both associated with Mφ/natural killer cell migration and interaction between T cells and dendritic cells [63]) are regarded as the most important chemokines in the fetal membranes [64], and their induction by LPS can be largely blocked by the activity of prolactin [41]. Chemokine transcript levels regulated more at the end of pregnancy include CCL13 (MCP-4, typically associated with Th2 type immune

227 responses [63]), and CCLs 19 and 21 (usually associated with lymph node homing by T
228 cells and DCs [63]), while some increase just at labor such as CXCL10 (a typical Th1
229 response chemokine, tightly regulated during pregnancy by decidual cells [65,66]) and
230 CCL8 (MCP-2, another typical Th2 response chemokine [63])[67]. Chemokine synthesis
231 during PPROM is dysregulated compared to term rupture of membranes, with IL-8 in
232 particular being consistently elevated [64]. Growth factors play important roles in
233 regulation of fetal size and communication between layers during PPROM, and can be
234 stored in regions of ECM, which would likely be released upon degradation of ECM
235 [11] and in response to cellular senescence signals [68]. Key growth factors within the
236 fetal membranes include granulocyte- Mφ colony stimulating factor (GM-CSF) [64,69]
237 (partially responsible for membrane weakening targeting the choriodecidua), vascular
238 endothelial growth factor [70] (having a large role in regulation of amniotic fluid
239 volume), and insulin-like growth factor (implicated in amnion epithelium proliferation
240 [71] and associated with PPROM [72]).

241

242 *Eicosanoids*: Eicosanoids are lipid mediators synthesized from arachidonic acid and are
243 converted to products including leukotrienes, thromboxane, and of particular
244 importance in the fetal membranes, prostaglandins [73,74]. These compounds serve
245 dual roles, both promoting inflammation and silencing key cytokines [74,75]. Of

246 relevance to the prostaglandins, COX's, PGE_2 and $PGF_{2}\alpha$ are widely expressed in the
247 fetal membranes[76,77]. PGE_2 is associated with parturition, and blockade of PGE_2 can
248 prevent the onset of some labor[78]. It is also associated with the suppression of Mφ
249 TNFα production[75]. In AMCs, NFκB induces PGE_2 synthase expression [79], and IL-
250 1β through NFκB is able to upregulate cyclooxygenase-2 [80]. In fact, within the
251 amnion, PGE_2 production is 22-32-fold higher from AMCs than AECs in response to
252 stimulation with arachidonic acid and cortisol [81].

253

254 *Extracellular vesicles and vesicular cargo* (proteins, microRNAs): Extracellular vesicles,
255 including the differently sized exosomes and microvesicles, are membranes released
256 from parent cells. They are considered a crucial mechanism of cell-to-cell
257 communication, and can be loaded with many types of cargo [82]. Extracellular vesicles
258 may carry a number of diverse cargo, including mRNA, proteins (particularly repressor
259 proteins such as suppressor of cytokine signaling (SOCS) proteins [83]), eicosanoids,
260 and microRNAs (miRNAs). MicroRNAs (miRNAs) are small non-coding RNAs that
261 help regulate gene expression within a cell through binding to mRNA and preventing
262 translation [84]. miRNAs can also serve as paracrine signaling molecules if packaged
263 into nanovesicles such as exosomes or other extracellular vesicles released from a cell of
264 origin and taken up by a target cell with cargo intact protected from the extracellular

265 environment [85]. While exosomes from CTBs [86] , amnion epithelium [87] and from
266 the DSC progenitor cell, the uterine stromal cell, have been characterized [88], there is to
267 date no research into decidual stromal cell-derived exosomes [88].

268

269 **Methods to study paracrine signaling in fetal membranes**

270 Culturing individual cell types in isolation limits understanding the biology of complex,
271 heterocellular environments such a tissues, whether in response to infection or in basal
272 state. However, there are various techniques available, and each has yielded valuable
273 insights. From simple histology to cutting-edge microfluidic, organotypic devices, we
274 have learned some fundamentals of where signaling originates and how it is
275 perpetuated throughout the tissue.

276 Methods to study different types of signaling between the membrane layers have
277 evolved over the past several decades. Each technique adds to our knowledge and
278 ability to understand paracrine signaling among the cell types of the fetal membranes.
279 Various histological methods can provide a sense of how the layers communicate by
280 determining which cells are making, or which are responding, to particular ligands,
281 such as sensing of microbial products via PRRs to initiate an immune response. By
282 histology, TLR 1, 2, 4, and 6 proteins are present in the DSCs, cytotrophoblasts, and
283 amniotic epithelial cells of the fetal membranes, suggesting that each of these cell types

284 is capable of detecting and signaling the presence of infection[89,90]. Prostaglandin E
285 synthase (PGES) expression is found in the amnion epithelium, fetal membrane Mφ,
286 and cytotrophoblasts[91], though DSCs are known to make large amounts of PGE_2 [75].
287 Inducible nitric oxide synthase, for the production of reactive nitrogen species, is found
288 in exclusively in mononuclear $CD14^+$ cells at the chorio-mesenchyme border and the
289 chorio-decidual border; an absence of endothelial nitric oxide synthase is observed in
290 cells of the fetal membranes with the exception of blood vessels at the decidua [92].

291

292 *Conditioned media experiments:* Transfer of supernatant (conditioned media) from one cell
293 type to another is a traditional way of determining what secreted factors may be
294 affecting the activity of neighboring cells. Early microarray analysis from Hess *et al.*
295 2007 showed that media from trophoblasts induced inflammatory and angiogenic
296 mediators from DSCs[93]. In more recent work, supernatant transfer from DSCs was
297 sufficient to suppress TNFα production in response to LPS from placental Mφs and
298 enhance the secretion of many other inflammatory mediators including IL-1β and IL-6
299 [75]. Blockade of PGE_2 production in DSCs rescued TNFα production, indicating that
300 DSCs suppress Mφ TNFα production via PGE_2. Conditioned media from amniotic
301 membranes was added to peripheral blood immune cell subsets and responses
302 characterized [94] and found that while T cells were skewed towards Th2 responses,

303 blood monocytes exhibited CXCL10 suppression but no change to levels of TNFα or IL-
304 6. Use of activated or resting Mφ-conditioned media on CTBs of the fetal membranes
305 induced changes in gene expression in leukocyte trafficking, adhesion, and cytokine-
306 related genes, all of which was independent of changes to DNA methylation and thus
307 likely not due to epigenetic regulation [95]. Recently, experiments performed on
308 separated choriodecidua and amnion found that conditioned medium generated from
309 choriodecidua treated with GM-CSF induced weakening of the amnion while treatment
310 of the amnion with GM-CSF alone did not, suggesting that factors secreted from GM-
311 CSF-stimulated choriodecidua result in weakening of the amnion [69]. Similar
312 experiments performed on separated choriodecidua and amnion found that
313 conditioned medium generated from choriodecidua containing IL-1α and β induced
314 production of human beta defensin 2 from amnion [96].

315

316 *Transwell systems:* Publications on direct co-culture of individual fetal membrane cell
317 types (primary or cell line) with either immune cells or other structural/parenchymal
318 fetal membrane cell types are limited. Shu *et al.* in 2015 employed a transwell setup to
319 determine the immune suppression of amnion mesenchymal cells on Mφs, showing
320 that the mesenchymal cells inhibited Mφ TNFα and IL-1β in a mechanism related to
321 ERK, JNK, and/or NFκB signaling [97]. In preliminary data from our laboratory, we

322 observed unique induction of CCL5 when placental Mφ were cultured with DSCs and
323 CTBs in a transwell system, and distinct levels of immunosuppression on Mφ
324 depending on whether the Mφ is in contact with the CTBs or the DSCs during infection.

325

326 *Tissue punches* To assess the function of the tissue as a whole, many labs use membrane
327 tissue punch biopsies (explants). To show altered capability in detecting infection,
328 Abrahams *et al.* conducted experiments showing differential modulation of TLRs
329 during infection with particular bacterial agents: *Mycoplasma* species increased
330 expression of TLRs 4, 6, and 8, while *E. coli* decreased expression of TLR10, and other
331 bacterial species had no effect (i.e. Group B *Streptococcus*) [47]. In complex cytokine
332 blocking experiments, Sato *et al.* in 2003 measured TNFα, IL-1β, IL-10, and PGE_2, then
333 selectively blocked each signaling molecule and determined the impact on secretion of
334 the others [98]. TNFα was required for maximal membrane production of IL-1β, IL-10,
335 and PGE_2, while blocking of IL-10 increased the amount of TNFα and PGE_2 produced.
336 However, the cells of origin of these cytokines cannot not be elucidated from this
337 system.

338

339 Roseblade, *et al.* in 1990 pioneered an *ex vivo* experimental model using tissue sections
340 secured to glass cylinders, effectively making a transwell using actual fetal membranes

341 as the porous membrane, making two compartments- the decidual side and the
342 amniotic side [99]. Through these experiments, they were able to determine that PGE_2
343 did not traverse the intact membranes easily from the amnion side to the decidual side.
344 This was followed up by Kent *et al.* in 1994 showing that cytokines added to the
345 decidual compartment, including TNFα, IL-1α, and IL-1β, were likewise only able to
346 cross from the decidua to the amnion in small quantities intact [100]. Zaga *et al.* in 2004
347 continued these fetal membrane transwell experiments by selectively infecting or
348 treating the amnion side or the decidual side, and they were able to deduce that the
349 decidual side secreted copious amounts of TNFα and IL-1β even when the amnion side
350 was the only infected area [101]. This demonstrated that amnion can communicate the
351 presence of infection to the decidua, although the mediators were unclear. Thiex *et al.* in
352 2009 then showed that LPS treatment to the choriodecidual side induced TNFα, IL-6, IL-
353 8, and IL-10 in the choriodecidual compartment, while the untreated amniotic side
354 secreted only TNFα [102]. In contrast, infection of the amniotic compartment induced
355 TNFα and IL-6 in the amniotic side, but TNFα, IL-6, and IL-8 from the choriodecidual
356 compartment, suggesting that the decidua fights infection without perturbing much of
357 the other membrane layers, but when infection reaches the amniotic side, inflammation
358 is bi-directional. Later experiments indicated that high levels of prolactin, such as that
359 found in 2nd trimester amniotic fluid, could suppress proinflammatory cytokine
360 production [103]. Work from Boldenow, *et al.* supported the findings with LPS, showing

361 that Group B *Streptococcus* infection in the choriodecidual compartment did not result in
362 infection of the amnion, and further stimulated production of the antimicrobial peptide
363 human beta defensin 2 [104].

364

365 *Mechanical methods of study:* Another method of investigating membrane cellular
366 interactions involves mechanical tests of membrane tensile strength. Membranes are
367 clamped in a stretching machine, one side fixed and the other attached to a spring
368 balance. The spring balance measures the stretching power in grams, which is
369 correlated to the percentage increase in amnion, total membranes, or uterine
370 muscle[105]. These tests of whole membranes resulted in broad changes to the
371 enzymatic and immune landscape of the membrane. Kumar *et al*. in 2006 performed
372 experiments using whole membranes and evaluating markers of tensile strength after
373 being subject to IL-1β or TNFα[23]. Treatment with TNFα or IL-1β induced collagen
374 remodeling (increased MMP9 and decreased TIMP3) and increased tissue markers of
375 apoptosis. IL-8 was induced most highly in whole stretched membranes, while
376 collagenase was induced most highly in stretched isolated amnion[105]. IL-8 appeared
377 to be produced most by the amniotic epithelial cells and the layer below the amnion,
378 and in intact membranes IL-8 was also produced by the reticular layer of the chorion
379 and the decidual stromal cells.

380

381 Separation of the layers and comparison of their individual responses to infection with
382 the response of the intact tissue also lends insight into how the layers may regulate each
383 other. Mechanically, the choriodecidua and amnion influence each other. Studies
384 investigating separation of the membrane layers showed consistent separation of the
385 amnion from the choriodecidua [106], and they had earlier shown that the work to
386 rupture fused amnion and choriodecidua was greater than the sum of the work to
387 rupture separated amnion and chorion [107]. ECM signaling pathways were found to
388 be greatly impacted by membrane rupture [108]. Sato *et al*. (2006) performed
389 experiments wherein they separated the chorion from the decidua and cultured the
390 explants, and found that relative to the intact choriodecidua, separated cultured chorion
391 and decidua produced more cytokine (TNFα, IL-10, IL-6) or PGE_2 individually than
392 intact choriodecidua [109], and Nhan-Chang *et al.* in 2010 further showed induction of
393 IL-6 and IL-8 in sites of membrane rupture [108], which are often accompanied by
394 spontaneous separation of amnion and choriodecidua [106,107]. This suggested to them
395 that auto or paracrine signaling networks feed back onto the layers to control
396 proinflammatory cytokine production when membranes are intact. However,
397 depending upon the mechanism of membrane separation and preparation, the
398 mechanical stress of physical separation of the layers can induce proinflammatory
399 cytokines [105].

400 *3D culture models:* As of yet, no 3D model of fetal membranes has been published. 3D
401 culture models are well-developed for placental biology[110-113]. However, a 3D
402 culture model of the fetal membranes has been less studied. Early work by Bilic *et al.* in
403 2005 utilized the amnion and mesenchymal layers of the membrane on a customized
404 scaffold of fibronectin, collagen I, and other components succeeded in mimicking many
405 aspects of the amnion, such as production of MMP9 in response to substrate [114].
406 Much research on 3D amnion culture models has since focused on the stem-like
407 properties of the mesenchymal stem cells of the amnion layer for the purposes of stem
408 cell therapy and tissue engineering and not for studies of fetal membrane paracrine
409 signaling, inflammation, or preterm rupture [115].

410 *Organ-on-chip:* Microfluidic, organotypic devices have been employed modeling many
411 organ systems and disease states, from tumor models to lymph nodes [116], livers [117]
412 and hearts [118], environmental toxins, and drug discovery [119,120]. To fully
413 recapitulate dynamic tissues *ex vivo*, microfluidic organ-on-chip (OoC) model systems
414 are being developed that better recreate the environment which cells of the fetal
415 membranes inhabit. Much has been written on the utility of these novel model organ
416 systems [121-124]. These models incorporate extracellular matrix, fluid dynamics,
417 contact with cell types of adjacent layers, and the presence of immune cells. Many of
418 these models have the potential to better investigate the paracrine signaling that exists
419 between various cell types of the fetal membranes.

420

421 Different groups have attempted to dissect the cell-cell communication between
422 characteristic cell types and cell layers in the endometrium[122,125] and in the
423 placenta[126-128]. Our laboratories developed and characterized the precursor to a
424 first-generation fetal membrane OoC using endometrial stromal cells and endothelial
425 cells from the uterus, which can found in Gnecco *et al.* 2017 [125]. The device consists of
426 two vertically-oriented chambers in a polydimethylsiloxane (PDMS) matrix separated
427 by a transparent resolution porous membrane (approx. 2 μm pore size) facilitating
428 media exchange between compartments and allowing for cell motility and bacterial
429 invasion between layers (Figure 2B). Umbilical vein endothelial cells and endometrial
430 stroma isolated from human tissues formed stable and confluent layers thanks to the
431 initial addition of Matrigel to the loading medium. In this work, the authors were able
432 to induce decidualization of the stromal cells mimicking a 28-day hormonal cycle,
433 essentially to recreate the uterine functions in the chip. The perfusion protocol
434 employed continuous laminar perfusion to mimic the hemodynamic forces, which were
435 shown to enhance decidualization as evidenced by increases in prolactin and IGFPB1
436 protein[129]. Our laboratory has since repurposed this microfluidic device (Fig. 2) as a
437 first-generation fetal membrane on chip: aiming to recreate the full structure of fetal
438 membranes, DSCs are seeded onto the bottom layer, CTBs on the top, and Mφs and
439 bacteria or bacterial product stimulation added to one or both compartments. While the

440 membranes separating each chamber prohibit cell-to-cell contact, they are permissive to
441 paracrine signaling between layers and better preserve the sheer stress and waste
442 disposal that the tissue naturally experiences. Further, the membranes are coated with
443 collagen to mimic the ECM of the membranes, and the pore size of the membrane (9
444 μm) is such that motile cells such as Mφ can migrate from one side to another, and there
445 is the possibility for in-growth at the membranous interface between the layers. In
446 ongoing work, our laboratory is performing experiments in a model of ascending
447 infection where the DSC (bottom) layer is infected with Group B *Streptococcus* and Mφ
448 activation within the DSC (bottom) and CTB (top) layer is being assessed to determine
449 the effect of CTB cell contact-mediated suppression of Mφ in response to signals from
450 an infected DSC layer. Thanks to transparency of the membrane and the
451 microfabrication process, this model is potentially scalable to produce 3 and 4-chamber
452 devices stacked vertically to add mesenchymal and amniotic epithelial cells, recreating
453 the cell-to-cell interactions of the whole fetal membranes. While the complexity of
454 assembly and fabrication of a multilayered device could be overcome by using
455 alternative polymers and bonding techniques[128], a vertical design presents limitations
456 in terms of compatibility with microscopy (due to the working distance of the optical
457 lenses) and of complexity to dissect events in the different compartments. Sensors (e.g.
458 glutamate, glucose, lactate, dopamine) on outflow pumps add greater utility to this

459 design, resulting in what is termed an "instrumented fetal membrane-on-chip"
460 (IFMOC).
461

462 Richardson *et al.* in 2019 published about their new OoC model for the amnion (AM-
463 OOC) where they modeled AMC and AEC in conjunction with collagen gel to mimic
464 the basement membrane[123]. In this device, microfluidic chambers are arranged in a
465 ring structure with an inner chamber surrounded by 2 lateral outer chambers and
466 connected by microchannels filled with type IV collagen (Figure 2A). The AM-OOC
467 facilitates investigation of cellular migration in amniotic membranes, something which
468 is insufficiently recapitulated with other cell culture methods. Communication between
469 layers can be observed by adding stimulus to one layer- in this case, compounds to
470 induce oxidative stress intended to mimic what is seen at term labor. During co-culture
471 in the AM-OOC, oxidative stress induced migration in both the AMC and AEC
472 populations, while mono-culture oxidative stress only induced migration in the AMCs,
473 an observation that would be missed without the AM-OOC co-culture system.
474

475 We have recently designed a planar microfluidic system, depicted in Fig. 2.c and in
476 greater detail in Fig. 3. This flat, single layered device presents several advantages in
477 terms of microfabrication, reduced thickness and simple scalability. Further, the planar

478 design allows for microscopy uncomplicated by depth of layers in the device found in
479 the vertical design, the absence of the physical barrier of a porous membrane, which
480 allows for more realistic ingrowth and cell-to-cell contact, and motility and paracrine
481 signaling less affected by gravitational forces skewing towards downward drift. Initial
482 validation of culture of human stromal cells and HUVECs with lipopolysaccharides
483 (LPS) separated by a thick barrier of collagen type I confirmed the possibility to detect
484 changes in IL-8 in response to a short bacterial exposure and to visualize morphological
485 changes during cell growth. LPS stimulation did not affect proliferation, but THESCs
486 showed a tenfold increase in IL-8 secretion at 24 hours after LPS stimulation. Following
487 the same stimulation with LPS, HUVECs produced less IL-8 than THESCs, exhibited a
488 threefold increase secretion with respect to the control.

489 Because there is no thick, dedicated collagen layer separating the chorion from the
490 decidua, current tests are now dedicated to the identification of the most appropriate
491 gel to recreate the extracellular matrix that characterize the fetal membrane at late
492 stages of gestation and to observe the invasion of trophoblasts and decidual cells into
493 the collagen, mimicking the interface of these two layers *in vivo*. A similar platform has
494 been used with placenta-derived cells and proposed the use of matrigel for the
495 mimicking the extravillous placental trophoblast cells into the uterine arteries during
496 placentation[130], however further studies are required to assess the compatibility of
497 the extracellular matrix with human cells and the potential effects on Mφ activation.

498 **Conclusion**

499 The technology to evaluate signaling between components of the fetal membrane is
500 emerging. As conditioned media experiments have confirmed, cell types of the fetal
501 membranes influence each other greatly[75,93,109]. The presence of infectious agents
502 likewise alters the constellation of surface receptors, as we see from histological
503 studies[51,90-92]. Culture in the presence of collagen in a 3D model system altered the
504 profile of MMP activity[114]. The culmination of each of these techniques is the organ-
505 on-chip, combining microscopy of multiple cell types within a single system, paracrine
506 signaling through secreted mediators, biologically relevant collagen to interrogate
507 mechanisms of membrane rupture, and precise control of infectious agents and immune
508 cells. These systems have the potential to yield novel insights into the steps leading to a
509 loss of membrane integrity and suggest new targets of intervention and biomarkers of
510 membrane breakdown.

511 Disclosure of interest

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516

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523

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870 **Figure legends**

871 **Figure 1. Cell types within the fetal membranes.** Layers of the fetal membranes and
872 approximate distribution of innate immune cells within membranes.

873

874 **Figure 2. Existing fetal membrane organ-on-chip device designs.** A) Amnion-on-chip
875 design by Richardson *et al.* is a central circle of amnion epithelial cells surrounded by a
876 concentric circle of mesenchymal cells and connected by collagen-filled microcapillaries.
877 B) 1st generation instrumented fetal membrane-on-chip derived from Gnecco *et al.*
878 uterus-on-chip, vertically oriented chambers separated by porous membranes, each
879 compartment with its inlet and outlet ports connected to chambers by microcapillaries.
880 C) 2nd generation instrumented fetal membrane-on-chip designed by Pensabene and
881 Mancini, horizontally oriented chambers separated by collagen.