Reversible EMT and MET mediate amnion remodeling during pregnancy and labor

Lauren S. Richardson1,2, Robert N. Taylor3, Ramkumar Menon1*

The amnion is remodeled during pregnancy to protect the growing fetus it contains, and it is particularly dynamic just before and during labor. By combining ultrastructural, immunohistochemical, and Western blotting analyses, we found that human and mouse amnion membranes during labor were subject to epithelial-to-mesenchymal transition (EMT), mediated, in part, by the p38 mitogen-activated protein kinase (MAPK) pathway responding to oxidative stress. Primary human amnion epithelial cell cultures established from amnion membranes from nonlaboring, cesarean section deliveries exhibited EMT after exposure to oxidative stress, and the pregnancy maintenance hormone progesterone (P4) reversed this process. Oxidative stress or transforming growth factor-β (TGF-β) stimulated EMT in a manner that depended on TGF-β–activated kinase 1 binding protein 1 (TAB1) and p38 MAPK. P4 stimulated the reverse transition, MET, in primary human amnion mesenchymal cells (AMCs) through progesterone receptor membrane component 2 (PGRMC2) and c-MYC. Our results indicate that amnion membrane cells dynamically transition between epithelial and mesenchymal states to maintain amnion integrity and repair membrane damage, as well as in response to inflammation and mechanical damage to protect the fetus until parturition. An irreversible EMT and the accumulation of AMCs characterize the amnion membranes at parturition.

INTRODUCTION

Human fetal membranes (collectively referred to as the amniochorion) are composed of multiple layers of cells and extracellular matrix (ECM). The amnion consists of an epithelial sheet oriented with the apical surface facing the amniotic cavity, a basement membrane rich in type IV collagen, and an underlying layer of fibroblasts. The chorion consists of trophoblast cells that are attached to the uterus, a basement membrane, and a layer of reticular cells. The amnion fibroblasts and the reticular layer of the chorion are attached to a spongy layer of ECM that separates the two membranes (1, 2). These mesenchymal cells embedded in ECM provide the structural scaffold for the avascular fetal membranes (3, 4), which define the intrauterine cavity and protect the fetus during gestation. The highly elastic amnion grows with the fetus from the time of embryogenesis and provides both immune and mechanical protection and endocrine functions (5). The amnion and chorion layers expand throughout gestation to accommodate the increasing volume of the fetus and amniotic fluid and fuse by the late first or early second trimester (6). Expansion of the fetal membranes requires constant physiological remodeling that is accompanied by cellular shedding (exfoliation) and repair of accumulated membrane microfractures (3). Failure to seal these microfracture defects can lead to degradation and loss of structural integrity of the membranes and appears to be a cause of preterm birth (2, 7).

Although ECM remodeling is reported to be mediated by matrix metalloproteinases (MMPs) (1, 8), the regulation of fetal membrane cellular remodeling is not well understood. As the innermost lining of the uterine cavity and a structural barrier, fetal membrane homeostasis is critical for successful maintenance of pregnancy. Senescence of fetal membrane cells also occurs as a physiological process that peaks near parturition because of mounting oxidative stress and inflammation in the intrauterine cavity at term, which is predominantly mediated by the p38 mitogen-activated protein kinase (MAPK) (9, 10). Aging of the fetal membranes can lead to parturition in both human and animal models (7, 11). Premature activation of fetal membrane senescence in response to various pregnancy-associated risk factors can predispose membranes to dysfunction and rupture. Preterm premature rupture of the membranes (pPROM) is a common complication of pregnancy and accounts for ~40% of spontaneous preterm births (12, 13). pPROM contributes to substantial neonatal morbidity and mortality, yet no diagnostic or clinical indicators exist to predict high risk. Current diagnostic tests only detect pPROM after its occurrence, creating a clinical quandary as to how to intervene for preventing preterm birth.

To understand predisposing factors that contribute to membrane weakening, specifically amnion stress and stretching, we investigated the mechanisms by which epithelial and mesenchymal cells in the amnion layer undergo remodeling or seal the gaps created by cell shedding. Amnion cells are capable of proliferation, migration, and pluripotent transitions, such as epithelial-to-mesenchymal transition (EMT) and the reverse, mesenchymal-to-epithelial transition (MET), during remodeling (14). Oxidative stress can prevent remodeling by increasing senescence and inflammation; by contrast, nutrient-rich amniotic fluid accelerates remodeling (14, 15). Two key factors that likely mediate cellular transitions during gestation are transforming growth factor-β (TGF-β) and progesterone (P4). TGF-β is an established promoter of EMT (16–18), and P4, the dominant pregnancy maintenance hormone, reduces EMT (19), in part, by promoting MET. We found that normal term parturition was associated with amnion membrane EMT, which also coincided with increased cellular and amniotic fluid TGF-β levels, in both humans and mice. We further tested the mechanisms of cellular transitions and the roles played by TGF-β and P4 and found that TGF-β binding to TGF-β receptor 1 (TGFBR1) led to the activation of the TGF-β–activated kinase 1 binding protein 1 (TAB1) complex, which promotes
autophosphorylation of p38 MAPK (20), thus stimulating amnion epithelial cell (AEC) EMT. We also identified a unique dual role for P4 in the membranes: P4 maintained the amnion epithelial phenotype by reducing the abundance of EMT-associated transcription factors in the mesenchymal cells and by forming a complex with P4 receptor membrane component 2 (PGRMC2), which induced MET in amnion mesenchymal cells (AMCs) through a mechanism that depended on the c-MYC proto-oncogene.

RESULTS
EMT is associated with human term labor
We compared morphological and molecular markers of EMT in amnion membranes from term labor (TL) vaginal deliveries to term not in labor (TNIL) membranes collected from scheduled cesarean deliveries. We first examined tight junctions in amnion membranes. Tight junctions are connections between epithelial cells, and EMT requires loosening of these junctions to enable epithelial cells to be shed and/or to migrate (21, 22). Migration also requires loosening of the collagen matrix (21). Transmission electron microscopy (TEM) showed smaller desmosome plaques and a significant reduction in tight junction length, accompanied by ECM collagen degradation in TL compared to TNIL amnion membranes (Fig. 1A). Masson’s trichrome staining also showed collagen degradation based on reduced intensity of blue staining marking the amnion membrane epithelium (Fig. 1B), thus supporting the TEM findings. Next, we examined the epithelium-specific marker cytokeratin 18 (CK-18) and the mesenchymal-specific marker vimentin (21, 23, 24). Consistent with our prior report that amnion cells normally exist in a metastate producing both intermediate filament types (7), dual immunohistochemical staining colocalized CK-18 and vimentin (Fig. 1B); however, CK-18 was higher in TNIL membranes, whereas vimentin was higher in TL membranes, suggesting an increase in EMT with labor.

We next examined the epithelium-specific adherens junction protein E-cadherin and its mesenchymal counterpart N-cadherin. Immunohistochemical analyses showed that TL amnion membranes had higher N-cadherin and lower E-cadherin compared to TNIL (Fig. 1B), confirming an EMT shift by AECs in labor. We also examined MMP9, one of the key type IV collagenases capable of degrading ECM (Fig. 1B). Recent studies by K. Sun’s group have shown that MMP7 (matrilysin) in amnion membrane cells can also degrade ECM, but this MMP was not tested here (25). MMP9, which is present in amniotic fluid during labor (1), was higher in TL compared to TNIL amnion membranes (Fig. 1B). To confirm histological data, Western blot analyses were performed and proteins were quantified (Fig. 1, C and D). TL amnion membranes showed higher (threefold) vimentin and a higher (2.4-fold) N-cadherin/E-cadherin ratio (Fig. 1, C and D, and fig. S5A). Labor was associated with an increase in the N-cadherin/E-cadherin ratio in favor of N-cadherin, consistent with increased EMT (26). The presence of specific transcription factors

Fig. 1. Human amnion membranes from normal term labor show evidence of EMT. (A) Transmission electron microscopy (TEM) of amnion membranes from TL (term labor) compared to TNIL (term not in labor) fetal membranes showing tight junctions (arrows). Insets show electron-dense desmosomes. Images are representative of three independent biological replicates. Scale bar, 0.05 nm. (B) Histological analyses of TL and TNIL membranes: Masson’s trichrome stain to show collagen, dual immunohistochemical (IHC) staining for CK-18 (pink) and vimentin (brown), and single IHC for N-cadherin, E-cadherin, and MMP9. Images are representative of three biological replicates. Scale bar, 50 μm. (C and D) Western blot analysis (C) and quantification (D) of TWIST, vimentin, N-cadherin, and E-cadherin in TL and TNIL membranes. Actin is a loading control. Cropped Western blots are shown; full blots are provided in the Supplementary Materials (fig. S5A). n = 5 biological replicates. Error bars represent means ± SEM. P = 0.004 (TWIST), P = 0.009 (vimentin), and P = 0.031 (N-cadherin/E-cadherin). Linear adjustment of contrast and brightness has been applied to the bright-field images throughout the figure. a.u., arbitrary units.


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associated with EMT—including TWIST, SNAIL, SLUG, ZEB1 (Zinc finger E-box–binding homeobox 1), and ZEB2—depends on the cell type, stimulus, and the time since stimulation. TWIST was significantly more abundant (27-fold increase) in TL than TNIL membranes (Fig. 1, C and D). Although increasing trends were seen with SNAIL, SLUG, and ZEB in TL, those data did not reach statistical significance (fig. S1A).

### Oxidative stress induces EMT in human amnion membranes

We used a highly validated in vitro organ explant system (27) to test whether the above findings were recapitulated in TNIL fetal membrane clinical specimens treated with cigarette smoke extract (CSE) to induce oxidative stress (17, 28, 29). Our prior work has shown that CSE treatment of explanted TL fetal membranes produces oxidative damage to proteins, lipids, and DNA that is identical to changes seen in TL membranes (30) and causes activation of the cellular stress mediator p38 MAPK (30, 31). CSE-treated fetal membranes in culture had a significant reduction in tight junction length as determined by TEM (Fig. 2A) and confirmed by Masson’s trichrome staining (Fig. 2B). As seen in TL fetal membranes, CSE treatment of TNIL membranes also induced higher levels of vimentin, N-cadherin, and MMP9 and lower levels of CK-18 and E-cadherin (Fig. 2, B to D). CSE also reduced the N-cadherin/E-cadherin ratio (1.5-fold) (Fig. 2D and fig. S5B). ZEB1 was significantly higher (8.3-fold) after CSE treatment compared to controls (Fig. 2, C and D), whereas SLUG and SNAIL were higher in response to CSE but did not reach statistical significance (fig. S1B).

### Oxidative stress induces p38-mediated EMT in a murine model

After observing EMT changes in human samples in vivo and in the organ culture in vitro model of oxidative stress, we proceeded to test whether similar changes could be identified in an animal model of labor. CD-1 mice were selected for examining EMT phenotypic changes in amniotic sacs on day 18 (D18), the penultimate day of pregnancy, versus D19, the morning of delivery in our colony. Masson’s trichrome staining showed that collagen was more degraded on D19 than on D18, and this was accompanied by higher levels of vimentin, N-cadherin, and MMP9 and lower levels of CK-18 and E-cadherin (Fig. 3A and fig. S6). Western blot analyses confirmed the histology data, with D19 showing significantly increased vimentin (3.3-fold change) and an increased N-cadherin/E-cadherin ratio (2.8-fold change) (Fig. 3B and fig. S6, A to C). SNAIL was significantly increased on D19 compared to D18 (6.9-fold change) (Fig. 3B), but changes in SLUG abundance did not reach statistical significance (fig. S1C). To test whether oxidative stress induced changes similar to those seen on D19, we injected CD-1 mice with CSE on D14 (29). Vimentin, N-cadherin, and MMP9 were higher, whereas CK-18 and E-cadherin were reduced (Fig. 3C). We previously reported that

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**Fig. 2.** TNIL human amnion membranes exposed to oxidative stress show evidence of EMT. (A) TEM of cultured TNIL amnion membranes either untreated or exposed to cigarette smoke extract (CSE). Tight junctions are noted with arrows. Insets show a close-up of one tight junction. Images are representative of three biological replicates. Scale bar, 0.05 nm. (B) Histological analyses of untreated TNIL and CSE-treated TNIL membranes: Masson’s trichrome stain to show collagen, dual IHC for CK-18 (pink) and vimentin (brown), and single IHC for N-cadherin, E-cadherin expression, and MMP9. Images are representative of three biological replicates. Scale bar, 50 μm. (C and D) Western blot analysis (C) and quantification (D) of ZEB1, vimentin, N-cadherin, and E-cadherin in untreated and CSE-treated TNIL membranes. Actin is a loading control. Cropped Western blots are shown; full blots are provided in the Supplementary Materials (fig. S5B). n = 3 biological replicates. Error bars represent means ± SEM. P = 0.036 (ZEB1), P = 0.664 (vimentin), and P = 0.0005 (N-cadherin/E-cadherin). Linear adjustment of contrast and brightness has been applied to all bright-field images throughout the figure.
oxidative stress causes protein peroxidation and p38 MAPK–mediated senescence in this model (29). To confirm that EMT induction in this model is associated with p38 MAPK activation, animals were coinjected with CSE and the p38 MAPK inhibitor SB203580 on D14, and tissues were collected on D18 for analysis. SB203580 reduced EMT, as measured by the epithelial and mesenchymal markers, whether delivered alone or in combination with CSE (Fig. 3C). SB203580 treatment also reduced pup loss and preserved placental weight (table S1).

Collectively, these data are supportive of an association among oxidative stress–induced p38 MAPK activation, EMT, and senescence to promote labor-associated changes in both humans and mouse. We cannot rule out that additive, indirect effects of gestational age may also contribute to these observations. The descriptive data detailed above used human TL and TNIL amnion membranes, mouse TL and TNIL amniotic sacs, and both in vitro (human) and in vivo (mouse) models of oxidative stress. We hypothesize that oxidative stress experienced at term before labor promotes p38 MAPK–mediated senescence and EMT in vivo. Furthermore, these studies suggest that senescence may be a key feature of EMT in fetal membranes, likely aided by oxidative stress–associated p38 MAPK activators.

### TGF-β induces TAB1- and p38-dependent EMT in AECs

We next addressed the possible endocrine, paracrine, or autocrine regulation of EMT using human primary AECs derived from normal TNIL amnion membranes. Other investigators have established that TGF-β induces AECs to undergo EMT (13, 32, 33), and we have shown that TGF-β, in response to oxidative stress, stimulates autophosphorylation of p38 through TAB1, which can bypass the apoptotic signal–regulating kinase 1 and MAPK kinase kinases (17). Therefore, we tested whether TGF-β–TAB1–p38 MAPK signaling might induce EMT in AECs. As a first step to determine whether this process could occur in vivo, we determined the amounts of TGF-β in human amniotic fluid samples from various pregnancy conditions. TGF-β was significantly increased in amniotic fluid from TL compared to TNIL (8.2-fold increase) and in the amniotic fluid of women with preterm birth with pPROM compared to women who had preterm birth with intact fetal membranes (20.8-fold change) (fig. S2A). Both TNIL and pPROM are associated with increased oxidative stress (23, 28, 29, 34–36) and p38 MAPK activation (17, 27–29, 37). These findings demonstrate that TGF-β...
knockdown and the pharmacological inhibitor of p38 MAPK, SB20358. Treatment with TAB1 siRNA reduced the abundance of TAB1 mRNA and protein (fig. S2C). TAB1 siRNA or SB203580 blocked the TGF-β-induced change from cuboidal to fibroblastoid cell morphology, which was quantified as cell shape indices (Fig. 4C and fig. S2D), supporting the hypothesis that TGF-β can trigger EMT in AECs in a manner that depends on TAB1 and p38. TGF-β treatment also increased SLUG and SNAIL abundance compared to controls (8.8-fold change and
findings of Feng (Fig. 4). Before testing the role of P4 in
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and migration during gestation. EMT is associated with inflammation,
transition is essential for filling gaps created by epithelial cell exfoliation
and oxidative stress (Fig. 4). AEC wound healing was accelerated by am-
vimentin staining (Fig. 3B). Cells at proliferative and migrating edges display
predominantly fibroblastoid shapes with superficial vimentin local-
ization, whereas cells at healed edges were cuboidal with perinuclear
vimentin staining (Fig. 4). AEC wound healing was accelerated by am-
niotic fluid and restrained by inducers of oxidative stress (Fig. 5). On the
basis of these lines of evidence, we postulate that MET facilitates the
reversal of fibroblastoid cells to an epithelial state because this tran-
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and sustained inflammation may damage or weaken fetal membranes.

The anti-inflammatory properties of P4 have been postulated to min-
and sustained inflammation may damage or weaken fetal membranes.
reducible by TAB1 siRNA or
and either TAB1 siRNA or
threefold change, respectively) (Fig. 4, D and E, and fig. S7, A and B).

Comet treatment of AECs with TGF-β and either TAB1 siRNA or
SB203580 reduced SLUG (5-fold and 21.2-fold, respectively) and
SNAIL (2.3-fold and ninefold, respectively) abundance compared to
TGF-β treatment alone. In accordance with this finding, we noted a
higher (2.7-fold) N-cadherin/E-cadherin ratio (Fig. 4E) in AECs
SB203580 cotreatment (twofold and twofold, respectively) (Fig. 4E).
SB203580 treatment alone did not induce changes in morphology,
N-cadherin/E-cadherin ratios, or SNAIL and SLUG abundance
(fig. S2E). In summary, these experiments are consistent with TGF-
β–induced, TAB1-mediated activation of p38 MAPK (17), leading
to increases in EMT-associated transcription factors and N-cadherin
and the adoption of a fibroblastoid cell shape (Fig. 4).

**Progestrone induces PGRMC2-mediated and c-MYC-mediated MET in AMCs**

EMT is a transitional state rather than a stable state, and a homeo-
static return of mesenchymal-to-epithelial phenotype is necessary for
the maintenance of fetal membrane integrity. A recent report from
our laboratory using scratch assays has shown that wounded
primary AECs resemble healing microfractures observed in intact
fetal membranes (7). This healing is associated with proliferation,
migration (EMT), and wound closure (EMT–MET switch) within
in 38 hours (7). Cells at proliferative and migrating edges display
predominantly fibroblastoid shapes with superficial vimentin local-
ization, whereas cells at healed edges were cuboidal with perinuclear
vimentin staining (7). AEC wound healing was accelerated by am-
niotic fluid and restrained by inducers of oxidative stress (7). On the
basis of these lines of evidence, we postulate that MET facilitates the
reversal of fibroblastoid cells to an epithelial state because this tran-
sition is essential for filling gaps created by epithelial cell exfoliation
and migration during gestation. EMT is associated with inflammation,
and sustained inflammation may damage or weaken fetal membranes.
The anti-inflammatory properties of P4 have been postulated to mini-
mize EMT’s inflammatory effects by transitioning cells back to their
epithelial state through MET (16). Before testing the role of P4 in
our in vitro system, we confirmed that AECs did not produce the
classical nuclear P4 receptors PRB and PRA, confirming the previous
findings of Peng et al. (9) and indicting that the actions of P4 in AECs
are likely mediated through the membrane P4 receptors PGRMC1
and/or PGRMC2. PGRMC1 abundance did not differ between human
TNIL and TL fetal membranes; however, we noticed a significant
labor-induced 3.2-fold decrease in PGRMC2 (figs. S3A and S7B).
This was recapitulated in vitro with AMCs subjected to CSE-induced
oxidative stress, showing no change in PGRMC1 but a threefold
reduction in PGRMC2 (figs. S3, B and C, and S7B). In addition,
overexpression of PGRMC2 in AMCs increased P4-induced MET,
and MET was evident even under oxidative stress conditions in cells
that overexpressed PGRMC2 (fig. S4, A to C).

On the basis of these data, we hypothesized that P4 induces MET
in AMCs through PGRMC2. We treated primary AMCs with P4 at
200 ng/ml, which approximates the concentration of P4 at term, for
6 days. P4 treatment was associated with a shift from fibroblastoid
to cuboidal shape with increased abundance of CK-18 (1.5-fold change)
(Fig. 5, A and B). We did not observe changes in vimentin staining.
Crystalline exclusion test showed that morphological changes in-
duced by P4 were not an artifact of changes in cell viability (fig. S3,
D and E). To test the role of PGRMC2 in this transition, we com-
pared EMT markers and cell morphology in P4-treated AMCs that
were either untreated or treated with a PGRMC2-specific siRNA
that reduced the abundance of both transcripts and protein (Fig. 5C
and fig. S3F). P4 treatment of AMCs led to a shift in epithelial mor-
phology that was blocked by knocking down PGRMC2 (Fig. 5C).
Western blot analysis showed a P4-induced increase (4.2-fold) in
c-MYC (Fig. 5, D and E), which has been reported to mediate the
effects of P4 signaling through PGRMC2 (39). P4 suppressed the
accumulation of EMT-associated transcription factors SNAIL (2.1-
fold change) and SLUG (4.5-fold change), and this was restored by
cotreatment with PGRMC2 siRNA (Fig. 5E). Furthermore, P4 treat-
ment decreased N-cadherin while increasing E-cadherin, contrib-
ting to an overall decrease in the N-cadherin/E-cadherin ratio
(5.7-fold change) (Fig. 5E). PGRMC2 siRNA reversed this change,
supporting a role for P4-PGRMC2 signaling in promoting MET.
We then tested whether P4-induced morphologic changes were
mediated by increased c-MYC. P4 treatment of AMCs led to a shift
in epithelial morphology that was blocked by the selective c-MYC
inhibitor 10058 (Fig. 6A), consistent with a role for c-MYC in
P4-induced MET. In support of this finding, cotreatment of P4 with
10058 showed trends, although not statistically significant changes,
of slightly higher SNAIL, SLUG, and N-cadherin/E-cadherin ratio
(Fig. 6, B and C). In summary, these experiments show that P4-
PGRMC2 elicited changes in cell morphology and decreases in
EMT-associated transcription factors and N-cadherin in a manner
that likely depended on c-MYC.

Treating AECs with P4 for 6 days had no effect on cell viability
(fig. S3G), maintained epithelial morphology (Fig. 7A and fig. S3H)
and CK-18 abundance, and significantly reduced vimentin compared
to untreated cells (1.7-fold change) (Fig. 7, A and B). In addition, P4
significantly increased c-MYC (2.4-fold change), decreased SNAIL
and SLUG abundance (2.3-fold and 7.7-fold change, respectively),
and reduced the N-cadherin/E-cadherin ratio (2.5-fold change) (Fig. 7,
C and D, and fig. S7A). These data support the theory that the high
concentrations of circulating P4 in late pregnancy maintain epithelial
characteristics in AECs.

**Reversible EMT and MET mediate amniotic membrane healing**

To further confirm that TGF-β and P4 conferred opposing effects
on AECs, we performed in vitro scratch assays. A scratch in an AEC
monolayer normally heals within 38 hours (Fig. 8, A and B). TGF-β
substantially inhibited the healing of AEC scratches, with full per-
sistence of the wound after 38 hours, whereas P4 accelerated healing
(Fig. 8A). Immunostaining for CK-18 and vimentin showed that
fibroblastoid-shaped cells with high amounts of vimentin dominated
the leading edges of wounds that failed to heal after 38 hours (Fig. 8B).
In contrast, healed areas after P4 treatment and in untreated cultures
were dominated by CK-18–positive cells with epithelial morphology
(Fig. 8B). These data further support the fact that P4-mediated re-
modeling of the amnion membrane is likely to promote membrane
repair and homeostasis during pregnancy, whereas oxidative stress–
induced TGF-β may induce a nonreversible state of EMT.

**DISCUSSION**

In this study, we investigated the mechanisms and mediators through
which epithelial and mesenchymal cells of the amnion membrane
undergo cyclic remodeling to maintain membrane integrity during
pregnancy. We showed that human and murine AECs exist in a
“metastate” at term, coexpressing both epithelial and mesenchymal markers (Figs. 1 to 3); however, mesenchymal features dominated in TL compared to TNIL fetal membranes. These findings were recapitulated in vitro with organ explants of TNIL fetal membranes that were exposed to oxidative stress (Fig. 2). These findings support previous reports by Janzen et al. (40) and Mogami et al. (10) that...
Amnion cells exposed to oxidative stress also undergo EMT. The transition from TNIL to TL is associated with increased oxidative stress, peroxidation of various cellular elements, and activation of p38 MAPK. Senescence and senescence-associated secretory phenotype (SASP; a form of sterile inflammation) have been observed in fetal membranes during labor (30). Fetal membranes at TL have more microfractures, degraded basement membranes, and augmented cell migration compared to not in labor fetal membranes (2). On the basis of our findings (Figs. 1 and 2), we speculate that molecular and cellular senescence is associated with a terminal (irreversible or static) state of EMT. EMT and associated inflammation and ECM matrix weakening are predisposing factors for mechanical disruption of amnion membranes prompting and facilitating labor and delivery.

We investigated the role of TGF-β as a facilitator of amnion EMT because TGF-β is increased in TL amniotic fluid compared to TNIL (Fig. S2A), immunoreactivity for TGF-β is higher in fetal membranes from TL versus TNIL (17), and TGF-β is one of the classical SASP markers (41). In addition, extracellular vesicles released from AECs under oxidative stress are enriched in TGF-β peptides (42), suggesting a paracrine pathway to promote EMT. Amnion cells exposed to oxidative stress also undergo TGF-β-mediated autophosphorylation of p38 MAPK (17), a key signal that enforces fetal membrane senescence. In summary, intrauterine oxidative stress at term can initiate a cascade of events mediated by TGF-β–p38 MAPK–senescence–EMT that will weaken the amnion. After EMT, the resultant mesenchymal cells are more susceptible to oxidative stress (43–45) than their epithelial predecessors, and they produce inflammatory cytokines and MMPs and stimulate collagen degradation that can compromise fetal membrane integrity (40). Loss of tight junctions also promotes disassembly of ECM scaffold structures (46). The accumulation of mesenchymal cells in TL versus TNIL membranes (Fig. 5A) supports this concept that the increased mesenchymal character of the constituent cells weakens the amnion, thus hastening membrane rupture and labor.

TGF-β–mediated EMT is a well-reported phenomenon across development, and our data indicate that the amnion epithelium is no exception. Blocking TGF-β–mediated signaling by silencing TAB1 reduced EMT-associated transcription factors and mesenchymal markers, thus maintaining epithelial characteristics. Silencing of TGF-β signaling also reduces p38 MAPK activation (17). Inhibition of EMT in AECs by treatment with a p38 MAPK inhibitor (SB203580) further implicates this pathway (Fig. 4). In both human fetal membranes and mouse amniotic sacs, the p38 MAPK inhibitor reduced mesenchymal intermediate filaments, junction markers, and transcription factors (Figs. 3 and 4). Although differential increases in ZEB (47), SNAIL (48), SLUG (49), and TWIST (50) were seen between species and distinct stimulants, all of these transcription factors can be activated by p38 MAPK to induce EMT. The potential redundancy may ensure the appropriate state of cellular transition. TGF-β–p38 MAPK signaling plays a major role in membrane homeostasis and cell remodeling throughout gestation (17) and is regulated by many factors including P4 (16, 40). AECs that undergo EMT but are not permanently shed may move into the mesenchymal layer where they remain as a pool of cells capable of repairing microfractures and gaps in the amnion membrane or recycling to the epithelium by undergoing MET. The production of reactive oxygen species (ROS) (27, 28) and p38 MAPK activation peak at term (23, 31, 34, 51, 52) to augment amnion cell senescence and
Fig. 7. P4 maintains the epithelial state of human AECs. (A) Bright-field microscopy showing morphology of primary human AECs treated with P4 for 6 days and confocal immunofluorescence showing vimentin and CK-18. Nuclei are labeled with DAPI. Images are representative of three biological replicates. Scale bars, 50 μm. (B) Quantification of CK-18 and vimentin in (A). Error bars represent means ± SEM. For vimentin, 223 ± 15.47 (control) and 130.1 ± 15.68 (P4); P < 0.0001. For CK-18, 523 ± 57.5 (control) and 480.3 ± 52.01 (P4); P = 0.166. n = 3 independent experiments. (C and D) Western blot analysis (C) and quantification (D) of the indicated proteins in untreated and P4-treated AECs. Cropped Western blots are shown; full blots can be found in the Supplementary Materials (fig. S7A). n = 5 biological replicates. Error bars represent means ± SEM. c-MYC (P = 0.0211), SNAIL (P = 0.023), SLUG (P = 0.007), and N-cadherin/E-cadherin ratio (P = 0.005). Linear adjustment of contrast and brightness has been applied to all bright-field images throughout the figure.

Fig. 8. P4 expedites wound healing of human AECs in vitro. (A) Healing of scratch wounds in primary human AECs in normal cell culture conditions (control) or treated with P4 or TGF-β. A red mask was applied to aid visualization of the wound field. Images are representative of three biological replicates. Scale bar, 100 μm. (B) Immunofluorescence showing vimentin (green) and CK-18 (red) in scratch-wounded primary AECs treated as indicated. A black mask was applied to aid visualization of the wound field. Images are representative of three biological replicates. Scale bar, 100 μm. Linear adjustment of contrast and brightness has been applied to all bright-field and fluorescent images throughout the figure.
SASP-induced inflammation to promote a terminal state of EMT (Fig. 9).

We also examined the mechanisms of recycling of mesenchymal cells back to an epithelial phenotype. Mesenchymal cells perform endocrine functions during gestation (53); however, these functions are tightly regulated and only require a limited number of cells (about 10% of amnion membrane cells are mesenchymal). Because mesenchymal cells are predisposed to generating inflammatory and ROS signals (43, 45), their numbers need to be tightly regulated, and this may be achieved by reprogramming them back to an epithelial state through MET. MET reestablishes cell-to-cell contacts, which are critical for maintaining membrane integrity. Because P4 is a known anti-inflammatory hormone that supports pregnancy maintenance, we tested its ability to reverse EMT to MET. We showed that P4, through PGRMC2, induces MET in a manner that depends on c-MYC (Figs. 5 and 6). Silencing PGRMC2 or inhibiting c-MYC increased mesenchymal transcription factors and promoted a fibroblastoid phenotype (Figs. 5 and 6). On the basis of these data, we postulated that P4 in the amniotic fluid or endogenously produced by AECs may play a functional role in maintaining membrane homeostasis. Because P4 increases PGRMC2 transcripts and protein in AMCs (fig. S3F), this also provides a feedforward loop to promote MET. Overexpression of PGRMC2 in AMCs increased P4-induced MET, and MET was evident even under oxidative stress conditions.

Fig. 9. Schematic of amnion membrane maintenance and disruption due to cellular transitions. The amnion membrane is composed of amnion epithelial cells (AECs) and amnion mesenchymal cells (AMCs) that are embedded in ECM and separated from the AECs by a basement membrane (BM) that is rich in type IV collagen. During gestation, progesterone (P4) helps maintain the epithelial state of AECs, and AMCs have a fibroblastoid morphology. We hypothesize that the plasticity of amnion membrane cells allows remodeling of the membrane through cycles of MET and EMT. This maintains inflammatory homeostasis during gestation through a balance of P4-mediated MET and TGF-β-mediated EMT pathways to limit localized inflammation and repair microfractures. At term and preterm, increased oxidative stress and senescence cause an increase in TGF-β both in AMCs and in amniotic fluid and a functional withdrawal of P4 signaling in AMCs due to a decrease in the P4 receptor PGRMC2. This induces a static state of EMT, increased numbers of AMCs, and inflammation, all of which contribute to labor-associated outcomes such as membrane rupture. TGFBR1, TGFβ receptor 1.
in cells that overexpressed PGRMC2 (fig. S4). However, PGRMC2 was reduced in TL fetal membranes, specifically in the mesenchymal cells (fig. S3A), likely because of oxidative stress in the amniotic cavity. Exposing AMCs in culture to CSE, an inducer of oxidative stress, caused a reduction in PGRMC2 (fig. S3B). No change in PGRMC2 was observed in AECs (fig. S3C), but a decrease was observed in PGRMC1, supporting prior reports by Feng et al. (9, 54). An overall reduction in PGRMC2 induced a localized “functional P4 withdrawal” that disrupted MET. We propose this as one of the mechanisms leading to a terminal or static state of EMT at term, indicating a role for P4 in maintaining fetal membrane integrity (Fig. 9).

Maintenance of fetal membrane integrity during gestation and its mechanical and functional disruption at term involve changes in both cells and matrix. Although collagenolysis is well reported, this study emphasizes the role played by the amnion cells themselves. AECs and AMCs can undergo cyclic reprogramming during gestation under the influence of changes in local tissue environment, such as localized inflammation promoting EMT and P4 supporting MET (Fig. 9). Increases in intrauterine oxidative stress, TGF-β- and p38 MAPK-mediated senescence, and EMT increase the number of AMCs in membranes. This can be detrimental to membrane homeostasis because AMCs are known inducers of prostaglandins, MMPs, and proinflammatory cytokines (55, 56), all of which can induce membrane weakening, leading to PROM. On the other hand, the accumulation of AMCs provides a mechanism to amplify fetal inflammatory responses that are required to promote parturition and therefore fulfill a natural and physiologic need at term. During gestation, P4, through its membrane receptor, controls the number of AMCs by converting them back to AECs (Fig. 9) to maintain membrane integrity. This study has some limitations because the amnion layer of the fetal membranes has dominated our focus. Microfractures also have been reported in the chorion layer (2), and chorion cells undergo a similar type of oxidative stress, p38 MAPK activation, and senescence (28). The chorion trophoblast cells and fibroblastoid cells of its reticular layer also can be susceptible to similar transitions. This region is vulnerable to signals from the maternal decidua and its resident immune cells (57). TGF-β and P4 were obvious choices for investigation in this study because the level of the former increases (17) and the activity of the latter is reduced (58) in TL. Furthermore, P4 is a known activator of c-MYC and an inducer of MET. Estrogens, interleukin-6, and other labor-associated growth factors known to increase in amniotic fluid at term, such as epidermal growth factor (EGF) and fibroblast growth factor, can also promote EMT (59–61). Although our model emphasizes the contribution of TGF-β, it is highly likely that EMT in TL is mediated by synergy and cooperation among multiple factors.

MATERIALS AND METHODS

Institutional review board approval

This study protocol is approved by the Institutional Review Board at The University of Texas Medical Branch (UTMB) at Galveston, TX as an exempt protocol to use discarded placenta after normal term cesarean deliveries (UTMB 11-251). No subject recruitment or consent was required for this study.

Clinical samples

Fetal membranes (combined amniocchorion and decidua) were collected from TNIL cesarean deliveries with no documented pregnancy complications and TL vaginal deliveries. Fetal membranes were dissected from the placenta, washed three times in normal saline, and cleansed of blood clots using cotton gauze. Six-millimeter biopsies (explants) were then cut from the midzone portion of the membranes, avoiding the regions overlaying the cervix or placenta. The amnion was then separated from the chorion, and explants were processed for a variety of assays.

Inclusion criteria are as follows: Normal term births were women with TL and delivery (>390/7 weeks) and no pregnancy-related complications. Exclusion criteria are as follows: Subjects with multiple gestations, placenta previa, fetal anomalies, and/or medical or surgeries (intervention for clinical conditions that are not linked to pregnancy) during pregnancy were excluded. Severe cases of pre-eclampsia or persistent symptoms (headache, vision changes, and right upper quadrant pain) or abnormal laboratory findings (thrombocytopenia, repeated abnormal liver function tests, creatinine doubling or greater than 1.2, or hemolysis, elevated liver enzymes, and a low platelet count (HELLP syndrome) or clinical findings (pulmonary edema or eclampsia) were excluded. Subjects who had any surgical procedures during pregnancy or who were treated for hypertension, preterm labor, or suspected clinical chorioamnionitis (reports on foul-smelling vaginal discharge, high levels of complement-reactive protein, and fetal tachycardia); who had positive group B streptococcus screening or diagnosis of bacterial vaginosis, behavioral issues (cigarette smoking and drug or alcohol abuse); and who delivered at term were excluded from the control groups.

In vitro fetal membrane organ culture and stimulation with CSE

The in vitro organ explant culture system for human fetal membranes and stimulation of membranes with CSE was performed as previously reported (27, 42, 62). In this study, CSE was used to mimic the oxidative stress experienced by fetal membranes at term before labor that will transition the membrane into a labor phenotype (30). Briefly, 6-mm biopsies of fetal membranes were collected from non-laboring cesarean deliveries and placed in an organ explant system for 24 hours. CSE was prepared by bubbling smoke drawn from a single lit commercial cigarette (unfiltered Camel; R. J. Reynolds Tobacco Co., Winston Salem, NC) through 50 ml of tissue culture medium [Ham’s F12/Dulbecco’s modified Eagle’s medium (DMEM) mixture with antimicrobial agents], which was then filter-sterilized through a 0.22-mm filter (Millipore, Bedford, MA) to remove contaminant microbes and insoluble particles. Fetal membranes were then stimulated with CSE (1:25 dilution) for 48 hours, whereas the cesarean explants were replaced with tissue culture medium. After a 48-hour treatment, the explants were removed, and the amnion was separated from the chorion and processed for a variety of assays.

Collection of CD-1 amniotic sacs

The Institutional Animal Care and Use Committee (IACUC) at the UTMB at Galveston approved the study protocol. CD-1 pregnant mice were purchased from Charles River Laboratories (Wilmington, MA). Animals were shipped on D10 of gestation and acclimated in a temperature- and humidity-controlled facility with automatically controlled 12-hour light/12-hour dark cycles. Mice were allowed to consume regular chow and drinking solution ad libitum. At day 14 of pregnancy, the pregnant CD-1 mice were weighed and subjected to minilaparotomy and injection of 150 μl of the treatment solution into uteri, physically between two to three gestational sacs, according to the following experimental groups: (i) CSE diluted in saline,

(ii) CSE in combination with SB203580 (p38MAPK inhibitor), and (iii) saline alone (control). After euthanizing the animals by using carbon dioxide inhalation according to the IACUC and American Veterinary Medical Association guidelines on D18, maternal, fetal, and placental weight was documented, and pup loss/reabsorption was counted. Amniotic sacs and placenta were collected in either 10% formalin or fresh-frozen in liquid nitrogen and stored at −80°C until further analysis. In addition, CD-1 mice at embryonic D18 and D19 of pregnancy were euthanized using carbon dioxide inhalation according to the IACUC and American Veterinary Medical Association guidelines. Amniotic sacs were collected in either 10% formalin or fresh-frozen in liquid nitrogen and stored at −80°C until further analysis.

AEC in vitro culture
Primary AECs were isolated from TNIL amnion (about 10 g), peeled from the chorion layer, and dispersed by successive treatments with 0.125% collagenase and 1.2% trypsin (17, 42). The dispersed cells were plated in a 1:1 mixture of Ham’s F12/DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), EGF (10 ng/ml), 2 mM l-glutamine, penicillin G (100 U/ml), and streptomycin (100 mg/ml) at a density of 3 million to 5 million cells per T75 and incubated at 37°C with 5% CO2 and 95% air humidity for 6 days. Cells were cultured to nearly 50% confluence, each flask was serum-starved for 1 hour; rinsed with sterile 1× PBS, followed by treatment with media (control), TGF-β (15 ng/ml) containing media, TGF-β + SB203580 (10 μM), a p38MAPK functional inhibitor, SB203580 alone, TGF-β + siRNA to TAB1 (150 nM), or progesterone (P4) (30 ng/ml); and incubated at 37°C with 5% CO2 and 95% air humidity for 6 days. To induce MET in AMCs, once cells reached 40 to 50% confluence, each flask was serum-starved for 1 hour; rinsed with sterile 1× PBS, followed by treatment with media (control), P4 (200 ng/ml) containing media, P4 + 10058 (75 μM), a pharmaceutical inhibitor of c-MYC, or P4 + siRNA to PGRMC2 (150 nM); and incubated at 37°C with 5% CO2 and 95% air humidity for 6 days. Cells were collected for polymerase chain reaction (PCR) and Western blots analysis. For siRNA transfection, to determine the potential role of TAB1 regulation of TGF-β–induced EMT in AEC and PGRMC2–induced MET in AMCs, we down-regulated TAB1 and PGRMC2 using siGENOME siRNA (GE Healthcare Dharmacon, Thermo Fisher Scientific) (table S2). Briefly, AECs and AMCs were cultured to nearly 50% confluence in DMEM/F12 medium supplemented with 10% FBS and antimicrobial agents (penicillin-streptomycin and amphotericin). Before siRNA transfection, cells were incubated with antimicrobial-free medium overnight. Next, cells were incubated for 4 hours with siRNA complexes, which were freshly prepared using either 150 nM siRNA to specific genes or nontarget siRNA as control and 0.3% Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM I Reduced Serum Medium. Cells were further incubated in growth media for 6 days. Down-regulation efficiency of the target genes was validated by quantitative reverse transcription PCR (qRT-PCR). Gene expression was normalized to nontransfected control.

AMC in vitro culture
AMCs were isolated from amnion membranes as previously described by Jin et al. (28) and Sato et al. (55) with slight modifications. Primary AMCs were isolated from amnion membranes of women experiencing normal parturient at term (not in labor) and undergoing a repeat elective cesarean section. Reflected amnion membranes from the chorion layer, and dispersed by successive treatments with 0.125% collagenase and 1.2% trypsin (17, 42). The dispersed amnion membrane solution was digested in digestion buffer containing Minimum Essential Medium Eagle (catalog no. 10-010-CV, Corning) for 1 hour at 37°C (water bath) to disperse the cells and remove the epithelial cell layer. The amnion membrane pieces were then washed three to four times using cold HBSS to inactivate the enzyme. The washed amnion membrane was transferred into a second digestion buffer containing Minimum Essential Medium Eagle (catalog no. 10-010-CV, Corning), collagenase type IV (1 mg/ml), and deoxyribonuclease I (25 μg/ml) and incubated in a rotator at 37°C for 1 hour. The digested amnion membrane solution was neutralized using DMEM/F12 media (catalog no. 16-405-CV, Corning), filtered using a 70-μm cell strainer, and centrifuged at 3000 rpm for 10 min. The cell pellet was resuspended in complete DMEM/F12 media supplemented with 5% heat-inactivated FBS (catalog no. 35-010-CV, Corning), penicillin G (100 U/ml), and streptomycin (100 mg/ml) (catalog no. 30-001-CV, Corning); plated at 3 million to 5 million cells per T75; and incubated at 37°C with 5% CO2 until they were 80 to 90% confluent.

Scratch assay and cell culture treatments
Passage 1 AECs were seeded at about 80% confluence in four-well coverslips and incubated at 37°C with 5% CO2 for 24 hours. AECs were then serum-starved for 1 hour, rinsed with sterile 1× PBS, and then scratched evenly down the middle of the well, in a straight line, with a 200-μl pipette tip (7). Cells were washed with sterile 1× PBS four times to remove any cell debris. Cell were then treated with TGF-β (15 ng/ml) or P4 (30 ng/ml) for 1, 24, and 38 hours. Bright-field and

Quantitation of AEC and AMC from amnion membranes
Fetal membranes were collected from TNIL cesarean deliveries with no documented pregnancy complications and TL vaginal deliveries. Fetal membranes were dissected from the placenta, washed three times in normal saline, and cleansed of blood clots using cotton gauze. Sections (0.0006 m²) were then cut from three different regions of the midzone, avoiding the regions overlaying the cervix or placenta. The amnion membrane was then separated from the chorion, and the sections were processed using the AMC collection method. AECs were removed after the first digestion, and AMCs were removed after the second digestion. Cells were counted manually and by using an automatic cell counter (Countess 2 FL Automated Cell Counter, Thermo Fisher Scientific). These data were then used for analysis to determine the changes in AEC/AMC ratios at TNIL and TL.

Cell culture treatments
To induce cellular transitions in AECs, once cells reached 40 to 50% confluence, each flask was serum-starved for 1 hour; rinsed with sterile 1× phosphate-buffered saline (PBS), followed by treatment with media (control), TGF-β (15 ng/ml) containing media, TGF-β + SB203580 (10 μM), a p38MAPK functional inhibitor, SB203580 alone, TGF-β + siRNA to TAB1 (150 nM), or progesterone (P4) (30 ng/ml); and incubated at 37°C with 5% CO2 and 95% air humidity for 6 days. To induce MET in AMCs, once cells reached 40 to 50% confluence, each flask was serum-starved for 1 hour; rinsed with sterile 1× PBS, followed by treatment with media (control), P4 (200 ng/ml) containing media, P4 + 10058 (75 μM), a pharmaceutical inhibitor of c-MYC, or P4 + siRNA to PGRMC2 (150 nM); and incubated at 37°C with 5% CO2 and 95% air humidity for 6 days. Cells were collected for polymerase chain reaction (PCR) and Western blots analysis. For siRNA transfection, to determine the potential role of TAB1 regulation of TGF-β–induced EMT in AEC and PGRMC2–induced MET in AMCs, we down-regulated TAB1 and PGRMC2 using siGENOME siRNA (GE Healthcare Dharmacon, Thermo Fisher Scientific) (table S2). Briefly, AECs and AMCs were cultured to nearly 50% confluence in DMEM/F12 medium supplemented with 10% FBS and antimicrobial agents (penicillin-streptomycin and amphotericin). Before siRNA transfection, cells were incubated with antimicrobial-free medium overnight. Next, cells were incubated for 4 hours with siRNA complexes, which were freshly prepared using either 150 nM siRNA to specific genes or nontarget siRNA as control and 0.3% Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM I Reduced Serum Medium. Cells were further incubated in growth media for 6 days. Down-regulation efficiency of the target genes was validated by quantitative reverse transcription PCR (qRT-PCR). Gene expression was normalized to nontransfected control.

Overexpression of PGRMC2 in AMCs
To determine whether oxidative stress (OS)–induced functional P4 withdrawal in AMCs occurs through PGRMC2, overexpression studies were carried out. AMCs were cultured to nearly 50% confluence in DMEM/F12 medium supplemented with 10% FBS and antimicrobial agents (penicillin-streptomycin and amphotericin) and then transfected with 800 ng of green fluorescent protein (GFP)–PGRMC2 expression plasmids (table S3) with FuGENE (1:3 plasmid weight) (Promega) in Opti-MEM I Reduced Serum Medium. After 24 hours, Opti-MEM was removed, and cells were treated with control, CSE (1:50), or CSE + P4 (200 ng/ml) for 48 hours.
confocal microscopy documented wound closure, morphology, and vimentin/CK-18 staining.

Transmission electron microscopy

Fetal membranes from cesarean and vaginal deliveries and fetal membrane explants from normal term pregnancies with or without CSE exposure were fixed, stained, and embedded in Poly/Bed 812. Initial fixation was for 24 hours at 4°C in a fixative with 2.5% paraformaldehyde, 0.2% glutaraldehyde, and 0.03% picric acid in 0.05 M cacodylate buffer. After fixation, samples were rinsed three times with cacodylate buffer and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer. Sonicated tissue was rinsed twice with deionized water and stained en bloc with 2% aqueous uranyl acetate for 1 hour at 60°C. The samples were then dehydrated by a series of ethanol-water solutions (50, 75, 95, and 100% ethanol for three exchanges). Dehydrated tissue was infiltrated with two exchanges of propylene oxide, then with propylene oxide–diluted Poly/Bed resin at 1:1 ratio and 1:2 ratio, and then twice with pure Poly/Bed 812. Last, the samples were embedded in Poly/Bed 812 and cured overnight at 60°C. Because precise tissue orientation could not be maintained during curing of the resin, the first resin blocks were cut to give a wide flat face for the desired sectioning plane, replaced into new embedding molds, and cured again. Samples were cut as 90–nm sections, placed on Formvar–coated slotted grids, and poststained for 3 min with a solution of Reynold’s lead citrate. Images were taken with a JEM-1400 electron microscope (magnification, ×4000 or ×1500; JEOL) (63). This value was used to calibrate the tight junction length from pixels to nanometers.

Bright-field microscopy

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

Confocal microscopy

Confocal microscopy images were captured using a Zeiss 880 confocal microscope (×10, ×40, and ×63) (Zeiss). Three random regions of interest per field were used to determine red (CK-18) and green (vimentin) fluorescence intensity. Uniform laser settings, brightness, contrast, and collection settings were matched for all images collected. Images were not modified (brightness, contrast, and smoothing) for intensity analysis. ImageJ software (National Institutes of Health; rsweb.nih.gov/ij; version 1.51J) was used to measure vimentin and CK-18 staining intensity from two focal plans of three different regions per treatment condition at each time point. Image analysis was conducted in triplicate for all cell experiments.

Immunohistochemistry

Human fetal membrane and mice amnion sac tissue sections were fixed in 4% paraformaldehyde for 48 hours and embedded in paraffin. Sections were cut at 5 μm thickness and adhered to a positively charged slide and attached by keeping them at 57°C for 45 min. Slides were deparaffinized using xylene; rehydrated with 100% alcohol, 95% alcohol, and normal saline (pH 7.4); and stained. Three images for each category were taken at ×10 and ×40 magnification. Images were processed with ImageJ, and staining intensity was measured in a uniform manner. The following anti-human/mouse antibodies were used for immunohistochemistry: vimentin (Abcam, ab92547), CK-18 (Abcam, ab668), N-cadherin (Abcam, ab98952), E-cadherin (Abcam, ab15148), MMP9 (Cell Signaling Technology, 13667), TGF-β (R&D Systems, MAB1835), and c-MYC (Cell Signaling Technology, D3N8F).

Trichrome staining for collagen

Tissue sections were fixed in 4% paraformaldehyde for 48 hours and embedded in paraffin. Sections were cut at 5 μm thickness and adhered to a positively charged slide and attached by keeping them at 57°C for 45 min. Slides were deparaffinized using xylene; rehydrated with 100% alcohol, 95% alcohol, and normal saline (pH 7.4); and stained using the Masson’s trichrome method to identify collagen components. The amnion epithelium was identified by a single layer of cells, whereas the ECM was identified as the area in between the amnion epithelium and the chorion layer. Three microscopic fields were captured at ×10 and ×40 magnification.

Protein extraction and immunoblotting

AECs, AMCs, and human and murine tissue were lysed with radioimmunoprecipitation assay lysis buffer [50 mM tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, and 1.0 mM EDTA (pH 8.0), and 0.1% SDS] supplemented with protease and phosphatase inhibitor cocktail and phenylmethylsulfonyl fluoride. After centrifugation at 10,000 rpm for 20 min, the supernatant was collected, and protein concentrations were determined using bicinchoninic acid (Pierce). The protein samples were separated using SDS–polyacrylamide gel electrophoresis on a gradient (4 to 15%) Mini-PROTEAN TGX Precast Gels (Bio-Rad) and transferred to the membrane using iBlot Gel Transfer Device (Thermo Fisher Scientific). Membranes were blocked in 5% nonfat milk in 1× tris-buffered saline–TWEEN 20 or in 5% bovine serum albumin (BSA) buffer for a minimum of 1 hour at room temperature and then probed (or reprobed) with primary antibody overnight at 4°C. The membrane was incubated with an appropriate secondary antibody conjugated with horseradish peroxidase (HRP) and immunoreactive proteins and then visualized using Luminata Forte Western HRP substrate (Millipore). The stripping protocol followed the instructions of Restore Western Blot Stripping Buffer (Thermo Fisher Scientific). No blots were used more than three times. The following anti-human/mouse antibodies were used for Western blot: N-cadherin (Abcam, ab98952), E-cadherin (Abcam, ab15148), vimentin (Abcam, ab92547), ZEB1 (Novus Biologicals, NBPI-05987), SNAIL (Abcam, ab180714), SLUG (Abcam, ab180714), TWIST (Abcam, ab175430), c-MYC (Cell Signaling Technology, D3N8F), PGRMC1 (Cell Signaling Technology, D6M5M), PGRMC2 (Thermo Fisher Scientific, PA5-59465), and β-actin (Sigma-Aldrich, A5441).

Quantitative RT-PCR

To determine the expression levels of TAB1 and PGRMC2 after treatments, cells were collected and lysed using lysis buffer (QIAGEN). RNA was extracted using an RNaseasy kit (QIAGEN) as per the manufacturer’s instructions. Total RNA (500 ng) was reverse-transcribed using a High-Capacity RNA-to-cDNA kit (Applied Biosystems). qRT-PCR was performed on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems) using Fast SYBR Green Master Mix (Applied Biosystems). The amplification thermal profile was 20 s at 95°C and 3 s at 95°C, followed by 30 s at 60°C (40 cycles). To confirm the presence of a single amplicon, a melt curve was carried out: 15 s at 95°C, 1 min at 60°C, 15 s at 95°C, and 15 s at 60°C. Changes in gene expression levels were calculated by using the ΔΔCt method. We used predesigned quantitative PCR assays from Integrated DNA Technologies (table S4).
Immunocytochemical localization of intermediate filaments cytokeratin and vimentin

Immunocytochemical staining for vimentin (3.7 µl/ml; Abcam, ab92547) and CK-18 (1 µl/ml; Abcam, ab668) were performed for multiple experimental end points (13). In addition, vimentin and CK-18 were measured during PGRMC2 overexpression studies. The manufacturer’s instructions were used to calculate staining dilutions to ensure uniform staining. After each time point, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked with 3% BSA in PBS before incubation with primary antibodies overnight at 4°C (38). This protocol is adequate to remove nonspecific binding of primary antibodies in our system. After washing with PBS, slides were incubated with Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies (Life Technologies), diluted 1:1000 in PBS, for 1 hour in the dark. Slides were washed with PBS, treated with Nuance Live ReadyProbes Reagent (Life Technologies), and then mounted using Mowiol 4-88 mounting medium (Sigma-Aldrich).

Crystal violet cell viability assay

To document cell viability after 6-day treatments, AECs and AMCs were seeded in a 12-well plate and treated as described above. After 6 days, cells were washed with 1× PBS, fixed with 4% paraformaldehyde for 15 min, washed with water, and stained with 0.1% crystal violet for 20 min. After 20 min, cells are washed and allowed to dry, and 10% acetic acid was added to each well. A 1:4 dilution of the colored supernatant was measured at an absorbance of 590 nm.

Cell shape index quantification

The cell shape index was determined for AEC and AMC cultures by evaluating one frame from each N (total of three images) per treatment for cell circularity using ImageJ software. The shape index was calculated using the following formula: SI = 4π × area/perimeter², which is an established method that was originally reported to determine vascular cell shape (64). A circle would have a shape index of 1; a straight line, an index of 0.

TGF-β enzyme-linked immunosorbent assay

Human amniotic fluid was collected at term vaginal deliveries (term labor), TNIL caesarian deliveries, pPROM, spontaneous preterm births, and a human/mouse TGF-β1 ELISA Ready-SET-Go! ELISA (second generation) (Affymetrix eBioscience) was conducted following the manufacturer’s instructions. Standard curves were developed with recombinant protein samples of known quantities. Sample concentrations were determined by correlating the samples absorbance to the standard curve by linear regression analysis.

Statistics

Statistical analyses for normally distributed data were performed using an analysis of variance (ANOVA) with the Tukey’s multiple comparisons test and t test. Statistical values were calculated using PRISM. P values of less than 0.05 were considered significant. Data are represented as means ± SEM.

SUPPLEMENTARY MATERIALS

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Fig. S1. EMT-associated transcription factors and p38 MAPK at term labor in human and mouse amnions.

Fig. S2. TGF-β1-associated changes in AECs.

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Acknowledgments: We thank L. F. Martin and J. Trivedi for collecting clinical samples and helping conduct immunohistochemistry and Western blots of fetal membrane explants. We would also like to thank B. Taylor for reviewing our raw data and statistical test for accuracy. Funding: L.S.R. is an appointed Pre-doctoral Trainee in the Environmental Toxicology (ETox) Training Program (T32ES007254), supported by the National Institute of Environmental Health Sciences (NIH) of the NIH of the United States and administered through the University of Richardsons et al., Sci. Signal. 13, eaay1486 (2020) 11 February 2020
Texas Medical Branch in Galveston, TX. This study is supported by the NIH/NICHD (1R03HD098469-01) to R.M. **Author contributions:** L.S.R. designed and conducted experiments, performed data analysis, and drafted the manuscript. R.N.T. helped with experimental guidance and manuscript preparation. R.M. conceived the project, designed experiments, helped with data analysis and interpretation, and prepared the manuscript. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

**Citation:** L. S. Richardson, R. N. Taylor, R. Menon, Reversible EMT and MET mediate amnion remodeling during pregnancy and labor. Sci. Signal. **13**, eaay1486 (2020).
Remodeling the amnion

During pregnancy, the amniotic membrane undergoes growth, repair, and remodeling processes that depend on epithelial-to-mesenchymal transition (EMT) and the reverse, MET. The membrane normally weakens just before parturition; aberrant weakening can lead to premature rupture. Richardson et al. found that amnions from mice and human term births exhibited increased EMT compared to amnions before the onset of labor and that oxidative stress stimulated EMT in preterm amnions. Oxidative stress and transforming growth factor-β (TGF-β), which are increased at the end of pregnancy, promoted EMT, whereas the pregnancy maintenance hormone P4 promoted MET. The authors propose that balanced EMT and MET maintain amnion homeostasis until the accumulation of oxidative stress and inflammatory factors trigger irreversible EMT, amnion weakening, and membrane rupture at parturition.