

One-Sentence Summary: Repairs to ruptures in the amniotic sac may involve EMT elicited by fetal macrophages.

Editor's Summary:

Fetal macrophages to the rescue

Premature rupture of the amniotic sac, which encloses the fetus, may trigger preterm birth. Because the amniotic sac may reseal, Kawamura *et al.* investigated repair mechanisms in the innermost epithelial cell layer of the amniotic sac, known as the amnion. The authors found that macrophages were recruited to rupture sites in human and mouse amnion, which showed signs of epithelial-mesenchymal transition (EMT), a process that confers mesenchymal attributes to epithelial cells and that is critical for tissue repair. Depletion of macrophages in fetuses in pregnant mice prevented macrophage recruitment, EMT, and repair of amnion ruptures. Thus, fetal macrophages induce EMT in the epithelial cells surrounding rupture sites in the amnion to facilitate repair.

Title: Fetal macrophages assist in the repair of ruptured amnion through the induction of epithelial–mesenchymal transition

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Abstract: The premature rupture of the amniotic sac, a condition referred to as a preterm prelabor rupture of membranes (pPROM), is a leading cause of preterm birth. In some cases, these ruptured membranes heal spontaneously. Here, we investigated repair mechanisms of the amnion, a layer of epithelial cells in the amniotic sac closest to the embryo. Macrophages migrated to and resided at ruptured sites in both human and mouse amnion. A process called epithelial-mesenchymal transition (EMT), in which epithelial cells acquire a mesenchymal phenotype and which is implicated in tissue repair, was observed at rupture sites. In dams bearing macrophage-depleted fetuses, the repair of amnion ruptures was compromised, and EMT was rarely detected at rupture sites. The migration of cultured amnion epithelial cells in wound-healing assays was mediated by EMT through TGF- β –Smad signaling. These findings suggest that fetal macrophages are crucial in amnion repair because of their ability to induce EMT in amnion epithelial cells.

Main Text:

INTRODUCTION

Preterm birth is a leading cause of perinatal morbidity and mortality (1). Preterm prelabor rupture of membranes (pPROM) is a main cause of preterm birth (2). Although approximately 30% of pPROM is caused by ascending infection (3), causes unrelated to infection include smoking, maternal undernutrition or psychological stresses, intrauterine bleeding during pregnancy, and iatrogenic causes such as amniocentesis, fetoscopy, and accidental rupture at the time of cervical cerclage (2). Rupture of the fetal membranes has been previously considered to be an irreversible process, and most women with pPROM begin labor spontaneously within several days. However, a small proportion of women with pPROM remain undelivered (2), and spontaneous repair of the membranes has been observed (4). The mechanism by which ruptured membranes repair is not known.

Fetal membranes are composed of three layers: amnion, chorion, and decidua (1). The primary load-bearing structure of the fetal membranes is the collagen-rich amnion (fetal tissue comprised of superficial epithelial cells and underlying mesenchymal cells). Amnion mesenchymal cells synthesize interstitial collagens that maintain mechanical integrity of the amnion.

Epithelial-mesenchymal transition (EMT) is a biological process in which epithelial cells differentiate and acquire a mesenchymal phenotype (5). EMT enhances migration, invasion, and synthesis of the extracellular components of epithelial cells. These epithelial cells are essential in embryogenesis, organ development, wound healing, and cancer metastasis and invasion (6), and EMT assists in tissue repair in various tissues (7, 8).

Macrophages play important roles in wound-healing processes (9, 10). Tissue-resident macrophages reside in various organs, such as the brain, lung, liver, spleen, and intestines, where they regulate homeostasis of resident tissues, patrolling for external pathogens (11). If a tissue is damaged, these resident macrophages immediately defend the tissue from noxious pathogens and repair the injury. The roles of macrophages in the developing fetal tissues when the fetal membranes are injured, however, remain unknown.

Here, we revealed the role of resident macrophages in the fetal membranes. Using mice in which fetal macrophages were conditionally depleted, we showed that these macrophages are necessary for wound healing of amnion and are crucial for induction of EMT in injured amnion epithelial cells.

RESULTS

Macrophages and EMT at the rupture site of human amnion

First, we histologically analyzed the rupture site of human fetal membranes in 6 pregnancies complicated by rupture of the fetal membranes (ROM) (Fig.1, A to D, fig. S1A-E, and Table 1). Macroscopic analysis of spontaneous pPROM without infection at 30 weeks of gestation and subsequent cesarean delivery revealed that the numbers of both amnion epithelial and mesenchymal cells were increased at the rupture site compared with the intact site (Fig. 1A). Although rarely observed in intact fetal membranes, CD68⁺ and CX3CR1⁺ macrophages were prevalent at the edge of rupture (Fig. 1B, C). These macrophages migrate to the site because the amnion is avascular. At intact amnion, CD86⁺ macrophages (M1-phenotype) were rare, and although their number was slightly increased in the rupture site, it was not statistically significant (Fig. 1D). In contrast, CD206⁺ macrophages (M2-phenotype) were increased in the rupture site (Fig. 1D). Specifically, most of the macrophages that migrated to the rupture site were CD206⁺ M2-phenotype macrophages.

Next, we sought to detect EMT in human fetal membranes. The epithelial marker E-cadherin was expressed in the monolayer of human amnion epithelial cells in intact fetal membranes, and the mesenchymal marker vimentin was expressed beneath the mesenchymal layer of amnion (Fig. 2A). At the rupture site, however, vimentin⁺ cells as well as N-cadherin⁺ cells were observed in the epithelial layer, and their numbers were increased in cases of ROM (Fig. 2A, B), suggesting that EMT occurs in human amnion. E-cadherin and cytokeratin 18 (CK18) were consistently expressed in the epithelium of the rupture site (100%), even in vimentin⁺ epithelial cells (Fig. 2A, C), suggesting that this change was partial (or intermediate) EMT. The overall number of cells in the amnion epithelial layer at the rupture site was increased significantly relative to intact fetal membranes (Fig. 1B). This finding was reinforced by the detection of Ki67⁺ cells in the injured epithelial layer, but not in intact membranes, suggesting that amnion epithelial cells proliferate only at the site of injury (fig. S1F). Collectively, these results suggest that when fetal membranes are damaged and ruptured, macrophages migrate to the rupture site of amnion where EMT and proliferation are induced. Tissue remodeling processes are therefore ongoing in the amnion.

Mouse model of sterile pPROM

Next, we assessed the healing of amnion in vivo using the mouse model of mechanical ruptured membranes (13). On 16 days post coitum (dpc) after laparotomy, fetal membranes were ruptured with a sterile needle. Macroscopically, perforation of the amnion was visible 0 and 24 h after rupture, but the rupture was almost repaired at 48 h (Fig. 3A). The wound size was markedly decreased from 24 h to 48 h (Fig. 3B). Histology revealed that the intact amnion was composed of a monolayer of epithelial cells and mesenchymal cells intermittently dispersed beneath the epithelial layer (Fig. 3C). In contrast, cell numbers were increased at the edge of the rupture site although the surface was covered by a monolayer of epithelial cells (Fig. 3C). Masson's trichrome staining revealed collagen deposition at the edge of healing amnion (Fig. 3D).

In intact mouse amnion, F4/80⁺ CX3CR1⁺ macrophages were rarely observed (Fig. 3E). In contrast, migration of macrophages to the rupture site was apparent (Fig. 3E). The number of F4/80⁺ CX3CR1⁺ macrophages was increased 4-fold at 24 and 48 h around the rupture site compared to that in intact amnion (Fig. 3E). In contrast to humans, the number of Ki67⁺ cells was not increased in the rupture site of amnion in mice (fig. S2). This suggests that the increased cells at the rupture site may be due to cell migration or EMT, not to proliferation.

We further analyzed whether EMT occurred at the rupture site in amnion in mice. In intact amnion, the monolayer of E-cadherin⁺ epithelial cells was distinct from the underlying vimentin⁺ mesenchymal cells (Fig. 4A). At the rupture site, however, E-cadherin⁺ vimentin⁺ double-positive cells appeared in the epithelial layers (Fig. 4A, B). Particularly at the site of repair, superficial E-cadherin⁺ amnion epithelial cells (AECs) were found within the thickened vimentin⁺ mesenchymal layer suggesting the possibility that AECs supplied these mesenchymal cells that were arrayed perpendicularly between epithelial and mesenchymal layers (Fig. 4B). Another epithelial marker, CK18, was expressed throughout both intact and rupture sites (Fig. 4C). Although the mesenchymal marker N-cadherin was rarely observed in intact epithelium, N-cadherin was increased in the rupture site (Fig. 4D). The presence of mesenchymal markers in the epithelium led us to explore TGF- β -Smad signaling (a pathway crucial for inducing EMT). Cytokines in the TGF- β superfamily bind to TGF- β 1 receptors which then activate intracellular Smad3 by phosphorylation (5). At the wound site, the number of phosphorylated Smad3⁺ cells was increased in the ruptured site of murine amnion whereas they were rarely observed in the intact amnion (Fig. 4E). Gene expression analysis revealed that *Tgfb1* mRNA was increased at the rupture site compared to intact fetal membranes (Fig. 4F). In contrast, *Tgfb2* and *Tgfb3*

mRNA were not increased (Fig. 4F). Collectively, these results suggest that rupture of murine amnion results in migration of macrophages to the injured site which may activate the TGF- β -Smad pathway and lead to partial EMT in AECs.

Generation of fetal macrophage-depleted mice

To validate the roles of macrophages in the wound repair in the amnion, we generated fetus-specific conditional knockout mice using the tamoxifen (TAM)-induced Cre-LoxP system (Fig. 5A). *Cx3cr1^{CreER/CreER} Csf1r^{flox/flox}* male mice were crossed with *Cx3CRI^{+/+} Csf1r^{flox/flox}* female mice, and the genotype of all fetuses was *Cx3cr1^{CreER/+} Csf1r^{flox/flox}* (denoted as “Cre⁺”). On 14 and 16 dpc, TAM was intraperitoneally injected into pregnant *Cx3CRI^{+/+} Csf1r^{flox/flox}* dams (Fig. 5B), and CSF1R in CX3CR1-expressing cells was deleted in fetuses but not in dams. As a control, we used fetuses with the *Cx3cr1^{+/+} Csf1r^{flox/flox}* genotype (denoted as “Cre⁻”), generated by crossing *Cx3CRI^{+/+} Csf1r^{flox/flox}* male mice with *Cx3CRI^{+/+} Csf1r^{flox/flox}* female mice. CSF1R in CX3CR1-expressing cells was not deleted in control fetuses.

First, we checked the status of macrophages in maternal tissues. In maternal lungs, the number of macrophages was not changed in TAM-injected Cre⁻ and Cre⁺ mice, as expected (Fig. 5C). Next, flow cytometry analysis showed that vehicle injection did not change the percentage of CD45⁺ macrophages that were CX3CR1⁺ in fetal membranes and lungs (Fig. 5D, fig. S3). However, TAM injection depleted the number of CX3CR1⁺ macrophages in Cre⁺ fetal membranes compared to Cre⁻ fetal membranes (Fig. 5D). In contrast, the number of CX3CR1⁻ macrophages was not changed by TAM injection in Cre⁺ fetal membranes (Fig. 5D). Similarly, the number of CX3CR1⁺ macrophages was decreased by TAM injection in Cre⁺ fetal lungs, but not in Cre⁻ fetal lungs (Fig. 5E). TAM injection also decreased *Csf1r* mRNA expression in fetal membranes and lungs (Fig. 5F). Fetal weights among the four groups were similar (Fig. 5G), and there were no aborted fetuses among the four groups. Together, these data indicated the successful generation of conditional CX3CR1⁺ macrophage-depleted fetuses.

Compromised amnion repairing in macrophage-depleted fetal membranes

To test amnion repair capacity in these macrophage-depleted ($M\phi^-$) fetuses, we ruptured fetal membranes at 16 dpc and collected tissues 24 h after rupture (Fig. 5B). In $M\phi^-$ fetuses (Cre^+ TAM), the rupture of the amnion was larger at 24 h compared to that in macrophage-replete ($M\phi^+$) fetuses (Cre^- TAM, Fig. 6A). The wound size at 24 h was larger in $M\phi^-$ fetuses compared to that in $M\phi^+$ fetuses (Fig. 6A). Repair of amnion was normal in $M\phi^+$ fetuses. Specifically, as seen in WT mice, the continuity of the amnion structure was restored and numerous mesenchymal-like cells and immune cells aggregated around the rupture site (Fig. 6B). Conversely, amnion structure was disrupted in $M\phi^-$ fetuses (Fig. 6B) and the amnion was thinner compared to $M\phi^+$ fetuses (Fig. 6B). Immunofluorescence analysis confirmed $CX3CR1^+$ $F4/80^+$ macrophages at the amnion rupture site in Cre^- TAM fetuses but not in Cre^+ TAM fetuses (Fig. 6C). This result suggests that the wound-repairing macrophages were of fetal origin rather than of maternal origin. Next, we determined if EMT of AECs during amnion repair was affected by loss of these macrophages. Consistent with WT amnion (Fig. 4A), E-cadherin⁺ vimentin⁺ cells appeared in the epithelial layer of the rupture site in $M\phi^+$ amnion, and amnion mesenchymal cells were localized to two or three cell layers beneath the epithelial lining (Fig. 6D). Vimentin⁺ cells were absent in the epithelial layer in $M\phi^-$ amnion (Fig. 6D). Whereas CK18 was present in both $M\phi^+$ and $M\phi^-$ amnion (Fig. 6E), the abundance of mesenchymal marker N-cadherin was decreased significantly in $M\phi^-$ amnion (Fig. 6E). Phosphorylated Smad3 was present in the epithelial layer of $M\phi^+$ amnion but not in $M\phi^-$ amnion (Fig. 6F). Further, *Tgfb1* and *Tgfb2* mRNA was suppressed in ruptured $M\phi^-$ fetal membranes (Fig. 6G). Vehicle injection did not improve wound repair of the amnion in $M\phi^-$ mice (Fig. 6A, 6C-F). Collectively, these results suggest that fetal macrophages are indispensable for amnion repair and that they are crucial for activation of TGF- β signaling and induction of EMT in AECs.

Macrophages enhance EMT in amnion epithelial cells

To investigate the interactions between amnion epithelial cells (AECs) and macrophages in vitro, fetal macrophages (isolated from human placenta; fM ϕ , Hofbauer cells) and adult macrophages (isolated from human adult peritoneal fluid; M ϕ) were used. In wound-healing scratch assays, iAECs co-cultured without direct contact with macrophages showed increased migration compared to those not cultured with macrophages (Fig. 7A, fig. S4A). Further, spindle-shaped

mesenchymal-like cells appeared at the wound edge whereas epithelial morphology was retained in the non-wounded, intact areas (fig. S4A). When AECs were co-cultured with macrophages such that two cell types had direct contact, AEC migration in wound healing assays was further accelerated (Fig. 7B, fig. S4B) and the number of vimentin⁺ AECs was increased (Fig. 7C, 7D, fig. S4C, S4D). These vimentin⁺ cells accumulated at the wound edge, suggesting the occurrence of EMT at the wound site. All AECs expressed the epithelial markers E-cadherin and CK18 in both intact cell layers and at wound edges (Fig. 7D, 7E, fig. S4D, S4E). Confocal microscopy at high magnification revealed that N-cadherin was not detected in AECs monocultures but was present on the edges of direct contact mixed cultures (Fig. 7F, fig. S4F). Collectively, these data suggest that macrophages accelerate migration of AECs through partial EMT (acquisition of mesenchymal markers with retention of epithelial characteristics). Expression of EMT-related genes was quantified in both macrophages and AECs. *TGFB* isoform mRNA expression did not increase in AECs or fetal and adult macrophages that were co-cultured without direct contact (fig. S5A, S6A). Further, mRNA levels of the EMT markers *CDH1* (which encodes E-cadherin), *CDH2* (which encodes N-cadherin), and *VIM* (which encodes vimentin) were not changed in AECs by co-culture with adult or fetal macrophages (fig. S5B, S6B). This could be due to EMT occurring mainly at the scratched edges and not in all AECs and therefore resulting in the dilution of the expression of mesenchymal marker genes during the analysis of all AECs in the culture dish. Finally, measurement of the concentration of TGF- β 1 in the medium showed that although basal synthesis of TGF- β 1 was greater in fetal and adult macrophages cultured alone than in AECs, it was not increased by co-culture with AECs (Fig. 7G, fig. S6C). In addition, immunoblotting revealed that relatively low TGF- β abundance in AECs was not increased by co-culture with fetal and adult macrophages (fig. S5C, S6D, S7). This finding suggests that macrophages enhance EMT in AECs through the release of TGF- β but that TGF- β synthesis by AECs is not augmented by macrophages or the presence of wounded AECs.

TGF- β 1 promotes EMT and migration in AECs

Finally, the effects of TGF- β 1 in AECs were analyzed. TGF- β 1 accelerated migration of AECs (Fig. 8A, 8B). Treatment with TGF- β 1 changed epithelial cell morphology to mesenchymal-like and induced lamellipodia-like features in cells at the wound edge (Fig. 8A). Treatment of AECs

with TGF- β 1 not only decreased expression of the epithelial marker *CDH1* but also increased the expression of mesenchymal markers *CDH2*, *VIM*, *SNAIL* (which encodes Snai Family Transcriptional Repressor 1), *SNAI2*, and *ZEB2* (which encodes Zinc Finger E-Box Binding Homeobox 2) (Fig. 8C). Moreover, TGF- β 1 increased the number of vimentin⁺-spindle-shaped cells in the intact, unwounded AEC monolayer and at the wound edge (Fig. 8D).

The TGF- β 1 inhibitor SB431542 blocked increases in AEC migration induced by co-culture with fetal or adult macrophages (Fig. 8E, fig. S6E). In proliferation assays, TGF- β 1 decreased the number of cells (Fig. 8F). Immunoblot analysis revealed that the treatment of AECs with TGF- β 1 resulted in the appearance of phosphorylated, nuclear Smad3 in as little as 5 min, the abundance of which peaked at 30 min whereas phosphorylated Smad3 was not detectable in untreated AECs at 30 and 60 min (Fig. 8G, fig. S7). In human amnion, the phosphorylation of Smad3 was increased in AECs around the rupture site relative to those in intact membrane (Fig. 8H). In the murine ruptured fetal membrane model, injection of SB431542 in each gestational sac after rupture of fetal membranes significantly compromised repair of amnion (Fig. 8I). Collectively, these results suggest that rupture of amnion results in fetal macrophage migration to the wound site and release of TGF- β 1, which activates the Smad3 pathway, EMT, and migration of AECs.

DISCUSSION

Here, we investigated mechanisms by which sterile rupture of amnion is repaired. We demonstrated that macrophages migrated to the rupture site in both humans and mice and were essential for repairing the amnion by inducing EMT in AECs (Fig. 9). The amnion possesses regenerating ability, and spontaneous repair of ruptured membranes mid-gestation or following a small puncture by amniocentesis has been reported (4, 14). However, a small injury by a punch biopsy of human fetal membranes does not undergo repair in vitro (15), suggesting that the fetal membranes alone were not able to repair and that other cells such as immune cells may be necessary. Our experiments indicated that fetal macrophages migrate to the damage site, are crucial for amnion repair, and induce EMT in AECs predominantly through TGF- β 1 signaling.

The pathogenesis of ROM is complex and may be due to mechanical force, infection, oxidative stress, and intrauterine hemorrhage, all of which may lead to fragile fetal membranes

and consequent rupture. Our simplified preclinical model of ruptured membranes mimicked one of the causes: mechanical rupture such as that in fetoscopy or fetal surgery. We sought to investigate amnion repair mechanisms under conditions without infection or chronic physical stresses. Johnston *et al.* reported a 15% sealing rate of ROM (4), and the American College of Obstetrics and Gynecology Practice Bulletin states that cessation of amniotic fluid leakage may occur in spontaneous pPROM (16). Thus, spontaneous repair is possible if intrauterine conditions are favorable for wound repair.

In addition to classical macrophages differentiated from circulating monocytes, which engage in phagocytosis against pathogens and apoptotic or necrotic tissues, there is another group of macrophages called tissue-resident macrophages (11). These resident macrophages are involved in various biological processes including thermogenesis in adipose tissue (17), erythrocyte disposal and iron recycling in the spleen and liver (18), synaptic pruning by microglia in the brain (19), limiting absorption of fungal metabolites by the epithelium in the distal colon (20), facilitation of electrical conduction (21), and supporting mitochondrial homeostasis in the heart (22). Tissue-resident macrophages are not restricted to the previous M1 or M2 classification due to their diverse functions (11). Here, we report that macrophages have a role in repair of ruptured amnion and that these macrophages express CX3CR1. The chemokine CX3CR1 mediates recruitment of macrophages at injured skin, which is necessary for skin wound healing (23). Use of CX3CR1-depleted fetal macrophages indicated that similar mechanism might apply to repair of the injured amnion. Resident macrophages were observed in the amnion of WT mice. *Csf1r*⁺ macrophages are of yolk sac origin (24), and these macrophages were depleted in our conditional KO mice, indicating that murine amnion-resident macrophages appear to be of yolk sac origin. In humans, however, the origin of amnion-resident macrophages is not known.

EMT of AECs has been reported by several authors. TGF- β in amniotic fluid, presumably synthesized in amnion, is increased by labor (25). EMT occurs in AECs exposed to oxidative stresses (26, 27) during pregnancy through endogenous production of TGF- β by AECs. Vimentin-positive AECs have been reported to be increased in the vaginally delivered amnion compared to that of cesarean delivery (28). Spontaneous EMT occurs in AECs by autocrine production of TGF- β (29), and AECs undergo TGF- β -dependent EMT shortly after being cultured (30). TGF- β is also synthesized in intrauterine tissues from trophoblasts and decidua of placental tissue (31). We and Menon's group previously showed that TNF and IL-1 β accelerate

the EMT of AECs in vitro and in vivo (13, 32). Our experiments results expand these findings and demonstrate that TGF- β released by macrophages played a central role in inducing EMT in amnion undergoing repair. Further, wounded amnion in macrophage-depleted fetuses do not undergo EMT, a process that is essential for tissue repair (33). The Snail family transcriptional factor Slug (also known as Snai2) regulates EMT, and *Slug*-knockout mice exhibit compromised cutaneous wound healing without evidence of EMT (34). EMT functions during wound healing to speed up epithelial cell migration and to supply the myofibroblasts that synthesize extracellular components and tissue remodeling. Cell migration is necessary to repair the injured tissue, so epithelial cells at the wounded edge are induced to undergo EMT. At the same time, mesenchymal-like cells aggregate at the wound edge in the amnion. These cells are presumably supplied by proliferating epithelial cells, because a substantial portion of myofibroblasts arise from epithelial cells undergoing EMT in the wound healing process (35). These proliferating mesenchymal-like cells secrete ECM proteins such as collagens, and they contribute to the reconstruction of the collapsed tissues. In regard to fetal membranes, we have previously suggested the potential of EMT in the healing of ruptured amnion in a preclinical mouse model (13). Therefore, EMT appears to be a survival technique for the amnion when fetal membranes are damaged.

In clinical situations, spontaneous healing of ruptured fetal membranes is rare. This is likely due to the numerous factors that inhibit amnion repair, such as inflammation from infection, a large wound, and continuous leakage of amniotic fluid through the rupture which destroys the scaffold needed for wound healing. Menon *et al.* proposed the concept of cellular senescence in fetal membranes (36). In general, senescent cells lose their proliferative and repair capacity, thus preventing tissue regeneration (37). Telomere length is shorter toward term and in the fetal membranes of pPROM cases than that from gestational age-matched preterm birth cases without ruptured membranes (36). We suspect that the senescent amnion of mid to late gestation has weak regenerative potency and spontaneous repair is rare whereas the rupture of fetal membranes due to diagnostic amniocentesis performed on the amnion earlier pregnancy is repaired (14). To overcome this problem, we are seeking better methods of utilizing wound-repairing macrophages. We conclude that amnion has high regenerative potential through EMT of AECs and that fetal macrophages are important in mediating this wound-repairing process.

These findings therefore provide new insights into potential future treatment of pPROM and prevention of preterm birth.

MATERIALS AND METHODS

Collection of human fetal membranes

Human fetal membranes were collected from six cases of spontaneous ruptured membranes with cesarean delivery (Table 1). We chose cases in which clinical chorioamnionitis was not observed upon placental pathological examination. The rupture sites of the fetal membranes were macroscopically identified (Fig. 1A, fig. S1A), and they were pinned down on the plate to properly spread the membranes. The tissues were then fixed with 4% paraformaldehyde overnight and kept in 70% ethanol until paraffin embedding. After fixation in paraffin, 4- μ m-thick sections were prepared for histology.

Generation of fetal macrophage-depleted mice

Cx3cr1^{CreER} mice (B6.129P2(Cg)-*Cx3cr1^{tm2.1}(cre/ERT2)*Litt/WganJ, Stock No: 021160) and *Csf1r^{fllox}* mice (B6.Cg-*Csf1r^{tm1.2Jwp}*/J, Stock No: 021212) were purchased from Jackson Laboratory and subsequently maintained and bred under standard housing conditions (14 h light/10 h dark cycle and fed ad libitum) in a specific pathogen-free room. All mice were on a C57BL/6J background. For macrophage depletion, *Cx3cr1^{CreER/CreER}* *Csf1r^{fllox/fllox}* male mice were mated with *Cx3cr1^{+/+}* *Csf1r^{fllox/fllox}* female mice. The fetal genotype from this cross was *Cx3cr1^{CreER/+}* *Csf1r^{fllox/fllox}* (Cre⁺). At 14 and 16 days post-coitum (dpc), 4 mg of TAM was intraperitoneally injected into the dams (Fig. 5B). Theoretically, the *Csf1r* gene of *Cx3cr1*-expressing cells in the fetus is deleted when TAM is injected because *Cre* recombinase is globally expressed in fetus. In contrast, the maternal *Csf1r* gene of *Cx3cr1*-expressing cells is not affected by TAM injection because all of the *Cx3cr1*-expressing cells lack *Cre* recombinase. As a control, *Cx3cr1^{+/+}* *Csf1r^{fllox/fllox}* male mice were mated with a *Cx3cr1^{+/+}* *Csf1r^{fllox/fllox}* female mice to generate fetuses with the genotype of all of the fetuses is *Cx3cr1^{+/+}* *Csf1r^{fllox/fllox}* (Cre-). Therefore, the *Csf1r* gene of *Cx3cr1*-expressing cells in both dams and fetuses is not deleted by TAM injection because all of these cells lack *Cre* recombinase. For both macrophage-depletion and control experiments, TAM or vehicle (Vehicle) was injected (Fig. 5B).

Mouse model of sterile ruptured membranes

C57BL/6J wild-type mice were purchased from SLC Japan. Pregnant mice were anesthetized with isoflurane at 16 dpc. A ventral incision was made and the fetal membranes were punctured through the uterus using a sterile 20-gauge needle. In each uterus, half of the gestational sacs were punctured (rupture group) and half were not (intact control). To mark the rupture sites, needles were soaked in 10% India ink before the puncture, which resulted in black staining of the edge of the punctured fetal membranes (Fig. 3A). After each procedure, the maternal abdomen was closed with 5-0 silk. At the time of sampling, the diameter of the rupture was measured using a digital caliper under a microscope. Where indicated, SB431542 (20 µg/g) or vehicle (ethanol) was injected between the uterus and the fetal membranes of each gestational sac immediately after rupture with a 20-gauge needle at 16 dpc. The diameter of the rupture was measured after 24 h. Fetal membranes were collected after washing with phosphate-buffered saline (PBS), quick frozen in liquid nitrogen, and stored at -80°C.

Immunofluorescent staining

Tissues were fixed in 4% paraformaldehyde overnight at 4°C and kept in 70% ethanol until paraffin embedding. Four micrometer sections were cut and stained. Sections were deparaffinized. Antigen retrieval was performed by incubation with proteinase K (P8107S, New England Biolab; working concentration, 0.6 units/mL) for 1 min at 37°C or by boiling in 10 mM sodium citrate buffer (pH 6.0) in a microwave for 20 min and cooling for 30 min at room temperature, or by boiling in Tris/EDTA buffer (10 mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0) in a microwave for 20 min and cooling for 30 min at room temperature (table S1). After antigen retrieval, sections were incubated with 10% normal goat serum (50062Z, Life Technologies) with 0.3% Triton X-100 for 30 min at room temperature. Tissue sections were incubated with a primary antibody in PBS with 1% BSA and 0.3% Triton X-100 at 4°C overnight. The primary antibodies used are shown in table S1. Tissue sections were next incubated with Alexa Fluor 488- or 594-conjugated secondary antibodies (Ig, heavy and light chains, Invitrogen, 1:500 dilution) in 10% normal goat serum for 1 h at room temperature. The sections were mounted with Prolong Gold Antifade Reagent with DAPI (P36935, Molecular Probes). Images were obtained by a Leica TCS-SP8 confocal microscope or a Keyence BZ-X800. Image J software was used to generate individual images. For quantification, the number of positive cells was counted in a blinded manner and divided by the number of DAPI⁺ total cells.

Cell suspension preparations

Murine fetal membranes and lungs were homogenized and incubated in PBS containing 1 mg/ml collagenase D (#11088858001, Roche) and 0.1 mg/ml DNase (#10104159001, Roche) for 1 h at 37°C, and were then passed through a cell strainer made of 100 µm nylon mesh to obtain a homogeneous cell suspension.

Flow cytometry

Data were acquired using a MACS Quant analyzer (Miltenyi Biotec) and analyzed using FlowJo software (Tree Star). Cells were stained with the following antibodies for 15 min at 4°C: CD45 (30-F11, BioLegend, #103115), F4/80 (BM8, BioLegend, #123107), and CX3CR1 (SA011F11, BioLegend, #149025). Non-viable cells were stained with 7-amino-actinomycin D (AAD) solution. The gating strategy used to identify CX3CR1⁺ or CX3CR1⁻ macrophages was as follows: CX3CR1⁺ macrophages, CD45⁺ F4/80⁺ CX3CR1⁺; CX3CR1⁻ macrophages, CD45⁺ F4/80⁺ CX3CR1⁻.

Isolation and culture of human AECs and macrophages

Separation and isolation of human AECs were performed as previously described (38). Briefly, amnion tissue was separated by blunt dissection and minced. Cells were dispersed by enzymatic digestion with trypsin (#27250-018, Gibco). Isolation of placental macrophages was performed as previously described with slight modification (39). Briefly, a placenta was obtained from an elective cesarean section without complication. Fetal membranes and decidua were removed, and approximately 20 g of placental fragments were washed with sterile PBS four times. Tissues were minced into 2 to 3 mm³ fragments and transferred to 4 sets of 25 mL of DMEM/F12 (#11581-15, Nakalai Tesque) with 1 mg/mL of collagenase (Collagenase B, #11088831001, Roche) and 0.1 mg/mL of DNase (#10104159001, Roche) in 50 mL Falcon tubes. Tissues were incubated for 75 min in a 37°C water bath by shaking. After digestion, tissues were filtered by metal sieves and 100 µm nylon mesh. Flow-through tissues were centrifuged at 300 g for 10 min. After removing supernatant, pellets were re-suspended in 10 mL of DMEM/F12. Two sets of 5 mL of Ficoll-Paque Plus (#17-1440-02, Cytiva) in 15 mL Falcon tubes were prepared, and the cell suspension was slowly overlaid on top before being centrifuged at 500 g for 30 min at 4°C. After centrifugation, the upper layer was removed with a pipette. The band at the intermediate

layer was collected and washed with DMEM/F12 (centrifuged at 300 g for 10 min, twice). Cells were seeded in a 75 cm² flask and adherent macrophages were used for experiments.

Human peritoneal macrophages were isolated from the lavage of peritoneal fluid from patients with benign gynecological diseases undergoing laparoscopic surgery. After centrifugation at 200 g for 10 min, cells were seeded on plastic culture dishes. After 2 h of incubation at 37°C, non-adherent cells were removed by washing with PBS. Adherent macrophages were used for experiments. Isolated amniotic epithelial cells and macrophages were harvested in DMEM/F12 with 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (#2625384, Nakalai Tesque). Cells were cultured in 5% CO₂ and 95% air at 37°C. Cell culture inserts (#353095, CORNING) were used when AECs were co-cultured with macrophages.

In the system in which cell types were cocultured using cell inserts, placental or peritoneal macrophages and AECs were harvested together when they reached confluence, so that the cell density ratio was 1:1. In mixed cultures, cells were seeded at a 5:1 ratio of AECs:macrophages (AECs: 5.0×10^5 cells/cm², macrophages: 1.0×10^5 cells/cm²).

RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from human AECs, macrophages, and the fetal membranes and lungs of mice using a PureLink RNA mini kit (#12183020, Invitrogen). Reverse transcription of RNA was performed using ReverTra Ace (#TRT-101, Toyobo Life Science). Primer sequences are shown in table S2. Quantitative real-time polymerase chain reaction (PCR) was performed using a Thunderbird SYBR qPCR mix (#QPS-201, TOYOBO Life Science) on a LightCycler 480 Real-time PCR system (Roche). All data were normalized using the indicated housekeeping gene.

Wound-healing scratch assay

Human AECs were plated on 96- or 24-well plates. At confluency, the center of each well was scratched with a 200 µl pipette tip. Ten ng/mL of TGF-β1 (#240-B, R&D Systems) or PBS, in the absence or presence of the TGF-β inhibitor SB431542 (10 uM, #13031, Cayman Chemical) was utilized in serum-free medium. The width of the wound was measured microscopically. Three different fields were selected in each well, and the width was averaged. Percent closure was calculated by dividing the width of the indicated time by the width of 0 h.

Proliferation assay

Cells were seeded on 96-well plates and treated with 10 ng/mL of TGF- β in serum-free medium. Proliferation assay was performed using a CellTiter-Blue cell Viability Assay (#G8080, Promega) according to the manufacturer's protocols.

Immunocytochemistry

Human AECs or macrophages were plated in 8-well chamber slides. Cells were fixed in 4% paraformaldehyde for 10 min. After washing with PBS, slides were incubated with 10% normal goat serum for 30 min at room temperature. Slides were incubated with primary antibodies overnight at 4°C. The primary antibodies used are shown in table S1. Slides were incubated with Alexa Fluor 488- or 594-conjugated secondary antibody (Invitrogen, 1:500) and mounted with Prolong Gold Antifade Reagent with DAPI (P36935, Molecular Probes). Images were obtained by Leica SP8 confocal microscope and generated by Image J software. For quantification, the number of marker-positive cells was counted in a blinded manner.

Nuclear protein extraction

Nuclear extraction from AECs was performed as described previously (40). Cells were harvested in 2 ml of cold PBS containing 0.6 mM EDTA and centrifuged at 5000 $\times g$ for 30 s. Cell pellets were gently resuspended in 800 μ l of cold Buffer A (10 mM Tris-HCl, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol) containing protease inhibitor (cOmplete Mini, #11836170001, Roche) and phosphatase inhibitor (phosSTOP, # 4906837001, Roche) and allowed to swell on ice for 30 min. Twenty five μ l of a 10% Nonidet P-40 solution was added to the cell suspensions, which were vortexed for 5 s. After centrifugation at 12,000 $\times g$ for 30 s, the supernatant was reserved as cytoplasm extracts. Nuclear pellets were resuspended in 150 μ l of cold Buffer B (20 mM Tris-HCl, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) containing protease and phosphatase inhibitors and rocked for 1 h at 4°C. After the removal of nuclear debris by centrifugation at 12,000 $\times g$ for 5 min at 4°C, nuclear extracts were stored at 4°C.

Immunoblots

Nuclear and cytoplasm extracts were loaded to an acrylamide gel immediately after extraction. In immunoblots of TGF- β , cells were lysed in RIPA buffer containing protease and phosphatase inhibitor cocktails. Samples were centrifuged at 10,000 g for 20 min and the supernatant was

loaded to an acrylamide gel. After SDS-PAGE, membranes were blocked with 5% BSA for 1 h at room temperature and incubated with primary antibodies overnight at 4°C (table S1). HDAC1 and β -actin were used as the loading controls for the nuclear fraction and cytoplasmic fractions, respectively. Blots were next incubated with a secondary antibody (Goat Anti-Rabbit IgG-HRP Conjugate, #170-6515, Biorad, 1:10000 or Goat Anti-Mouse IgG-HRP Conjugate, #170-6516, Biorad, 1:10,000) at room temperature for 1 h. Signal were detected by chemiluminescence (Pierce ECL Plus Western Blotting Substrate, #32132, Thermo Scientific). Images were captured using ChemiDoc XRS+ (Bio-Rad Laboratories).

Enzyme-linked immunosorbent assay (ELISA)

TGF- β 1 concentration in the conditioned media was measured by Quantikine ELISA kits for human TGF- β 1 (DB100B, R&D Systems) according to the manufacturer's instructions.

Statistical analysis

Values were expressed as means $\pm$ standard error of the mean (SEM) or standard deviation (SD). Comparisons of parametric data between the two groups were performed using Student's *t*-test, and comparisons among more than three groups were performed using one-way analysis of variance (ANOVA). Comparisons of non-parametric data between the two groups were performed using two-tailed Mann-Whitney U test, and comparisons among more than three groups were performed using Kruskal-Wallis test followed by Dunn's multiple comparison test. Non-parametric data of paired human samples (Fig. 1, B to D) was analyzed by Wilcoxon matched-paires signed rank test. Statistical analyses were performed using GraphPad Prism 6. *p*-values less than 0.05 were regarded as statistically significant.

Study approval

Human tissues were obtained in accordance with the Kyoto University Graduate School and Faculty of Medicine, Kyoto University Hospital Ethics Committee (G1149) after obtaining written informed consent from participants prior to inclusion in the study. Animal experiments were approved by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University (MedKyo 20149).

Supplementary Materials

488 Figs. S1-S7

489 Tables S1-S2

490 References and Notes

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Figure Legends

Fig. 1 Macrophages at the rupture site of human amnion

(A) Hematoxylin-eosin (H&E) staining of the intact and ruptured sites of the human amnion of a representative ROM case (30 weeks of gestation) with cesarian delivery. Bars, 50 μ m. (B-D) Right: representative immunofluorescence images for CD68 (B), CD68 and CX3CR1 (C), CD206 and CD86 (D), and DAPI for the case in (A). Bars, 50 μ m. Graphs showing the percentage of CD68⁺ (B), CD68⁺ CX3CR1⁺ (C), CD86⁺ (D), and CD206⁺ (D) cells per DAPI⁺ cells in field or epithelium. n = 6 ROM cases. Ruptured amnion was rolled at fixation, and the edge is indicated by an yellow arrow. Epi: epithelial, Mes: mesenchymal layer of amnion. n.s. indicates not significant. **, $p < 0.01$, ***, $p < 0.001$ using Wilcoxon matched-pairs signed rank test.

Fig. 2 EMT at the rupture site in human amnion

(A-C) Right panels: representative immunofluorescence images for vimentin and E-cadherin (A), N-cadherin (B), CK18 (C), and DAPI staining of the case described in Fig. 1A. Bars, 50 μ m. Left graphs showing the percentage of vimentin⁺ (A), and N-cadherin⁺ (B) cells per DAPI⁺ cells in field or epithelium. n = 6 ROM cases. The edge is indicated by an yellow arrow. Epi: epithelial, Mes: mesenchymal layer of amnion. *, $p < 0.05$, **, $p < 0.01$ using Wilcoxon matched-pairs signed rank test.

Fig. 3 Mouse model of sterile ROM

(A) Representative macroscopic view of murine ruptured fetal membranes at 0 h, 24 h, and 48 h. White arrows indicate a rupture site that was stained with India ink. Bars, 2 mm. (B) Diameter of the ruptured amnion at 24 h and 48 h; n = 12–15 fetal membranes from 4 pregnant mice in each group. (C) Representative H&E staining of intact and ruptured amnion at lower magnification (upper panels) and higher magnification (lower panels). Black arrows indicate the

rupture site. Bars, 50 μ m. **(D)** Representative Masson's trichrome stain of intact (left) and ruptured (right) sites. Bars, 50 μ m. **(E)** Representative immunofluorescence images of F4/80, CX3CR1, and DAPI in the intact and ruptured amnion. Bars, 50 μ m. Graphs showing the number of F4/80⁺ CX3CR1⁺ cells in the intact and ruptured amnion at 24 h and 48 h; n = 9 fields from 3 fetal membranes from 3 pregnant mice in each group. Am: amnion. Each graph shows means $\pm$ SEM of the values obtained. **, $p < 0.01$ using two-tailed Mann–Whitney U test.

Fig. 4 EMT in murine ruptured amnion

(A–E) Representative immunofluorescence images of vimentin and E-cadherin (A, B), CK18 (C), N-cadherin (D), phospho (p)-Smad3 (E), and DAPI of intact and ruptured amnion. Graphs showing the number of vimentin⁺ E-cadherin⁺ cells per total DAPI⁺ cells in epithelium (A), N-cadherin⁺ cells per total DAPI⁺ cells in epithelium (D), phospho-Smad3⁺ cells per total DAPI⁺ cells in epithelium (E); n = 9–12 fields from 3–4 fetal membranes from 3–4 pregnant mice in each group. **(F)** mRNA expressions of *Tgfb* isoforms in intact and ruptured fetal membranes; n = 5 fetal membranes from 3 pregnant mice in each group. Epi: epithelial, Mes: mesenchymal layer of amnion. Bars, 50 μ m. Graphs show means $\pm$ SEM of the values obtained. n.s. indicates not significant. *, $p < 0.05$, **, $p < 0.01$, using two-tailed Mann–Whitney U test.

Fig. 5 Generation of fetal macrophage–depleted mice

(A) Schematic for the generation of fetal macrophage (M ϕ)–depleted mice using the CreERT-mediated loxP-deletion system. **(B)** Time course of the experiment. **(C)** Representative immunofluorescence images of F4/80, CX3CR1, and DAPI in maternal lungs (dam: *Cx3cr1*^{+/+} *Csf1r*^{flx/flx}). Left: fetal genotype is *Cx3cr1*^{+/+} *Csf1r*^{flx/flx} (Cre- TAM, control fetuses), right: fetal genotype is *Cx3cr1*^{CreER/+} *Csf1r*^{flx/flx} (Cre⁺ TAM, M ϕ -depleted fetuses). Bars, 50 μ m. Graph: the number of F4/80⁺ CX3CR1⁺ cells in the maternal lungs per total DAPI⁺ cells; n = 9 fields from 3 mice per group. **(D, E)** Flow cytometry analysis of fetal membranes (D) and fetal lungs (E). Proportion of M ϕ is expressed as a percentage of total CD45⁺ leukocytes. Vehicle or tamoxifen (TAM) was injected into dams in which the fetal genotype was *Cx3cr1*^{+/+} *Csf1r*^{flx/flx} (Cre-) or *Cx3cr1*^{CreER/+} *Csf1r*^{flx/flx} (Cre⁺); n = 9 fetal membranes from 3–5 pregnant mice in

each group. **(F)** *Csf1r* mRNA expression in the fetal membranes and fetal lungs; n = 4 fetal membranes or lungs from 3 pregnant mice in each group. **(G)** Fetal weights among the four groups; n = 6–14 fetuses from 3–7 pregnant mice in each group. Graphs show means $\pm$ SEM of the values obtained. n.s. indicates not significant. *, $p < 0.05$, **, $p < 0.01$, using two-tailed Mann–Whitney U test.

Fig. 6 Repair of ruptured amnion is compromised in fetal macrophage-depleted mice

(A–E) Representative images of *Cx3cr1*^{+/+} *Csf1r*^{flx/flx} fetal membranes (Cre⁻ TAM, M ϕ ⁺, left) or *Cx3cr1*^{CreER/+} *Csf1r*^{flx/flx} fetal membranes (Cre⁺ TAM, M ϕ ⁻, right) were obtained 24 h after rupture (17 dpc). **(A)** Macroscopic view of the rupture site. Bars, 1 mm. Graph: diameter of the rupture in vehicle- or tamoxifen (TAM)-treated fetuses in which the fetal genotype is *Cx3cr1*^{+/+} *Csf1r*^{flx/flx} (Cre⁻) or *Cx3cr1*^{CreER/+} *Csf1r*^{flx/flx} (Cre⁺). n = 14–23 punctures from 3 pregnant mice in each group. **(B)** Hematoxylin-eosin (H&E) staining of the ruptured site. Graph: thickness of the ruptured and healed site. n = 5–6 fetal membranes from 3 pregnant mice in each group. **(C–F)** Representative immunofluorescence images for F4/80 and CX3CR1 (**C**, green and red, respectively), vimentin and E-cadherin (**D**, green and red, respectively), phospho-Smad3 (**E**, green), N-cadherin and CK18 (**F**, green and red, respectively) and DAPI (blue) of ruptured amnion (Am). Graph: F4/80⁺ CX3CR1⁺ cells per total DAPI⁺ cells (**C**), vimentin⁺ cells per total DAPI⁺ cells in epithelium (**D**), phospho-Smad3⁺ cells per total DAPI⁺ cells in epithelium (**E**), N-cadherin⁺ cells per total DAPI⁺ cells in epithelium (**F**). n = 9–16 fields of 3–4 fetal membranes from 3 pregnant mice in each group for (**C**) to (**F**). Bars, 50 μ m. **(G)** mRNA expression of *Tgfb* isoforms in ruptured fetal membranes. n = 4 fetal membranes from 4 pregnant mice in each group. Graphs show means $\pm$ SEM of the values obtained. n.s indicates not significant. *, $p < 0.05$, **, $p < 0.01$, using two-tailed Mann–Whitney U test.

Fig. 7 Fetal macrophages induce the EMT in human AECs

(A) Schematic showing how AECs were cultured alone or co-cultured with fetal macrophages derived from placenta (fM ϕ) in a separate compartment. Representative images from the wound healing scratch assay at 0 h and 48 h showing AECs cultured alone (left) or co-cultured with

fMφ (right). Bars, 200 μm. Dotted lines indicate the edge of the scratched area. Graph: the percent coverage of the scratched area as a function of time. AECs alone (black line) or co-cultured with fMφ (red line). n = 6 biological replicates per group, 3 independent experiments. **(B)** Schematic showing how AECs were cultured alone or mixed with fMφ. Representative images from the wound-healing scratch assay at 0 and 24 h showing AECs alone (left) or mixed with fMφ (right). Bars, 200 μm. Graph: the percent coverage of the scratched area at 24 h. n = 6 biological replicates per group, 3 independent experiments. **(C-E)** Representative immunofluorescence images of CD68 and vimentin (C), vimentin and E-cadherin (D), CK18 (E), N-cadherin (F) and DAPI in scratched AECs (left) or AECs mixed with fMφ (right).. Bars, 200 μm (C), or 50 μm (D-F). Graphs: the number of vimentin⁺ cells per total DAPI⁺ cells (C), N-cadherin⁺ cells per total DAPI⁺ cells in the wound edge (F). n = 9 biological replicates per group, 3 independent experiments. **(G)** TGF-β1 concentration in the conditioned medium from AECs cultured alone, fMφ cultured alone, or both cell types co-cultured together. n = 6 biological replicates per group, 3 independent experiments. Graphs show means ± SD of the values obtained. n.s indicates not significant. **p* < 0.05, ***p* < 0.01 using the two-tailed Mann–Whitney U test (A, B, C, F), or Kruskal-Wallis test followed by Dunn’s multiple comparison test (G).

Fig. 8 TGF-β1 promotes the EMT and migration in AECs

(A) Representative images of the wound-healing scratch assay with AECs treated with vehicle or TGF-β1 (10 ng/mL). Insets: enlarged images of the areas. Bars, 200 μm. **(B)** The percent coverage of the scratched area as a function of time. n = 6 biological replicates per group, 3 independent experiments. **(C)** Quantitative PCR analysis of EMT-related gene expression in AECs treated with TGF-β1 (10 ng/mL). n = 6 biological replicates per group, 3 independent experiments. **(D)** Representative immunofluorescence images of vimentin and DAPI in AECs subjected a wound-healing scratch assay and treated with TGF-β1 (10 ng/mL) at 48 h. Dotted lines indicate the edge of a scratch. Bars, 200 μm. Graph: the number of vimentin⁺ cells per total DAPI⁺ cells. n = 12 biological replicates per group, 3 independent experiments. **(E)** Schematic of the migration assay using the TGF-β1 inhibitor SB431542 (10 μM). AECs were cultured alone, co-cultured with fetal macrophages (fMφ), or co-cultured with fMφ and SB431542. Graph: the percent coverage of the scratched area at 24 and 48 h. n = 6 biological replicates per group, 3

independent experiments. **(F)** Proliferation assay of AECs treated with TGF- β 1 (10 ng/mL) or control. $n = 6$ biological replicates, 3 independent experiments. **(G)** Immunoblots of phospho-Smad3 and Smad3 in nuclear and cytoplasmic fractions from AECs treated with TGF- β 1 (10 ng/mL). HDAC1 was used as a loading control for the nuclear fraction and β -actin was used as a loading control for the cytoplasmic fraction. Representative of 3 independent experiments. See also fig. S7. **(H)** Representative immunofluorescence images for phospho-Smad3 and DAPI of the intact and ruptured human amnion (Am). Dotted lines indicate the amnion. Bars, 50 μ m. Graph: the percentage of phospho-Smad3⁺ cells per DAPI⁺ cells in epithelial cells in the 6 cases of ROM. **(I)** Schematic showing how SB431542 (20 μ g/g) or vehicle was injected between fetal membranes and uterus in each gestational sac. Graph: diameter of the ruptured amnion at 24 h ; $n = 8-12$ fetal membranes from 4 pregnant mice in each group. n.s. indicates not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using two-tailed Mann–Whitney U test (**B-D, F, I**), Kruskal–Wallis test followed by Dunn’s multiple comparison test (**E**), or Wilcoxon matched-pairs signed rank test (**H**).

Fig. 9 Model of macrophage-mediated healing of ruptured amnion

When sterile rupture of amnion occurs, fetal macrophages migrate to the injured site and TGF- β 1 released from these macrophages induces EMT in amnion epithelial cells by activating the TGF- β -Smad3 pathway, which accelerates the migration of epithelial cells and the wound healing of amnion.

Table 1. Six cases of human ruptured fetal membranes.

Cases	Maternal age (years old)	Parity	Gestational age at ROM (weeks)	Gestational age at delivery (weeks)	Days from ROM to delivery	Chorioamnionit is by pathologist	Complications
1	39	M	30	30	0	-	Diabetes mellitus, angina pectoris
2	32	M	33	33	0	-	Breech presentation
3	27	P	28	29	4	-	
4	40	M	31	31	2	-	Dichorionic diamniotic twin
5	33	M	35	35	1	-	Myasthenia gravis
6	30	P	38	38	0	-	Breech presentation

ROM: ruptured fetal membranes, M: multiparous, P: primiparous.

Fig. 1

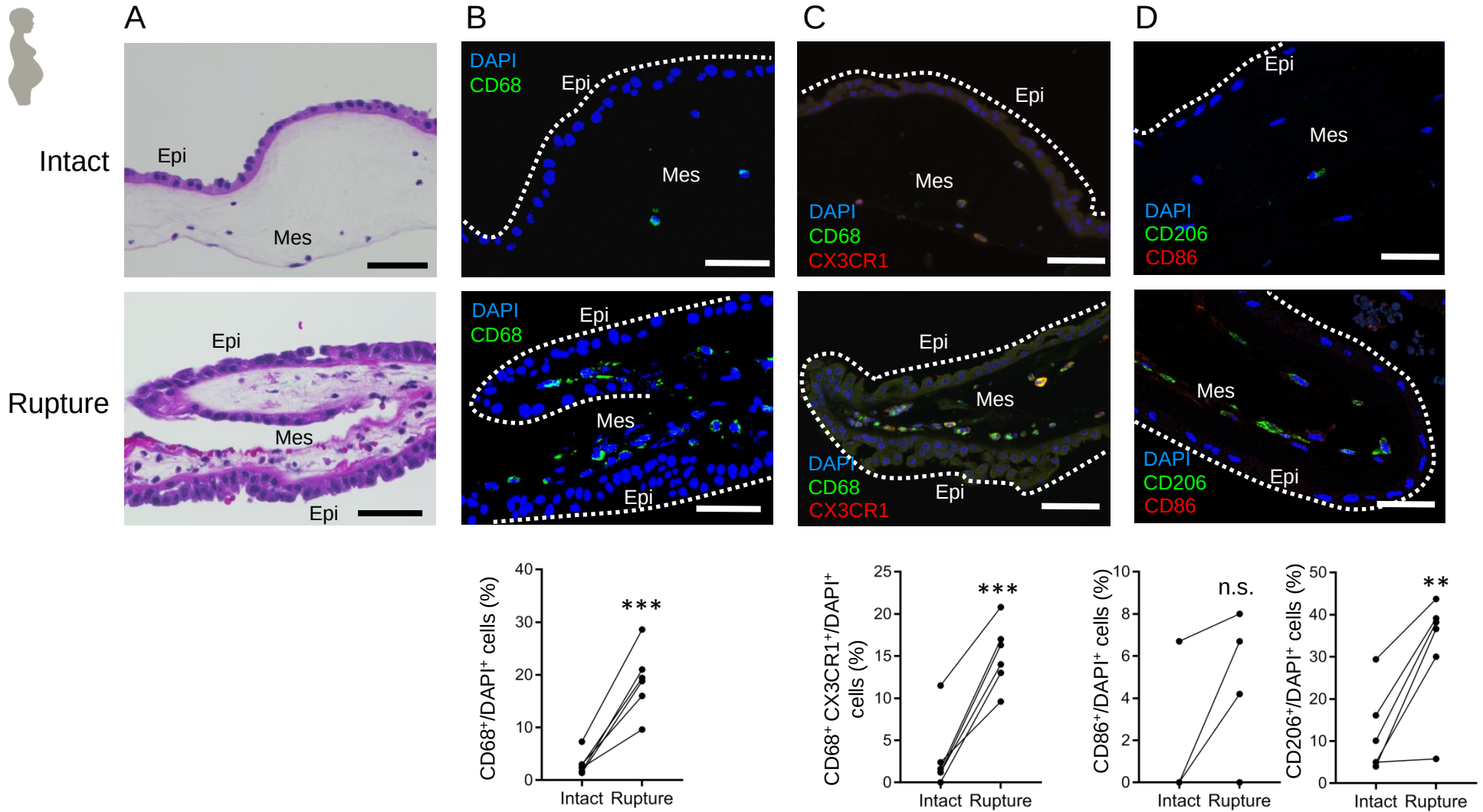


Fig. 2

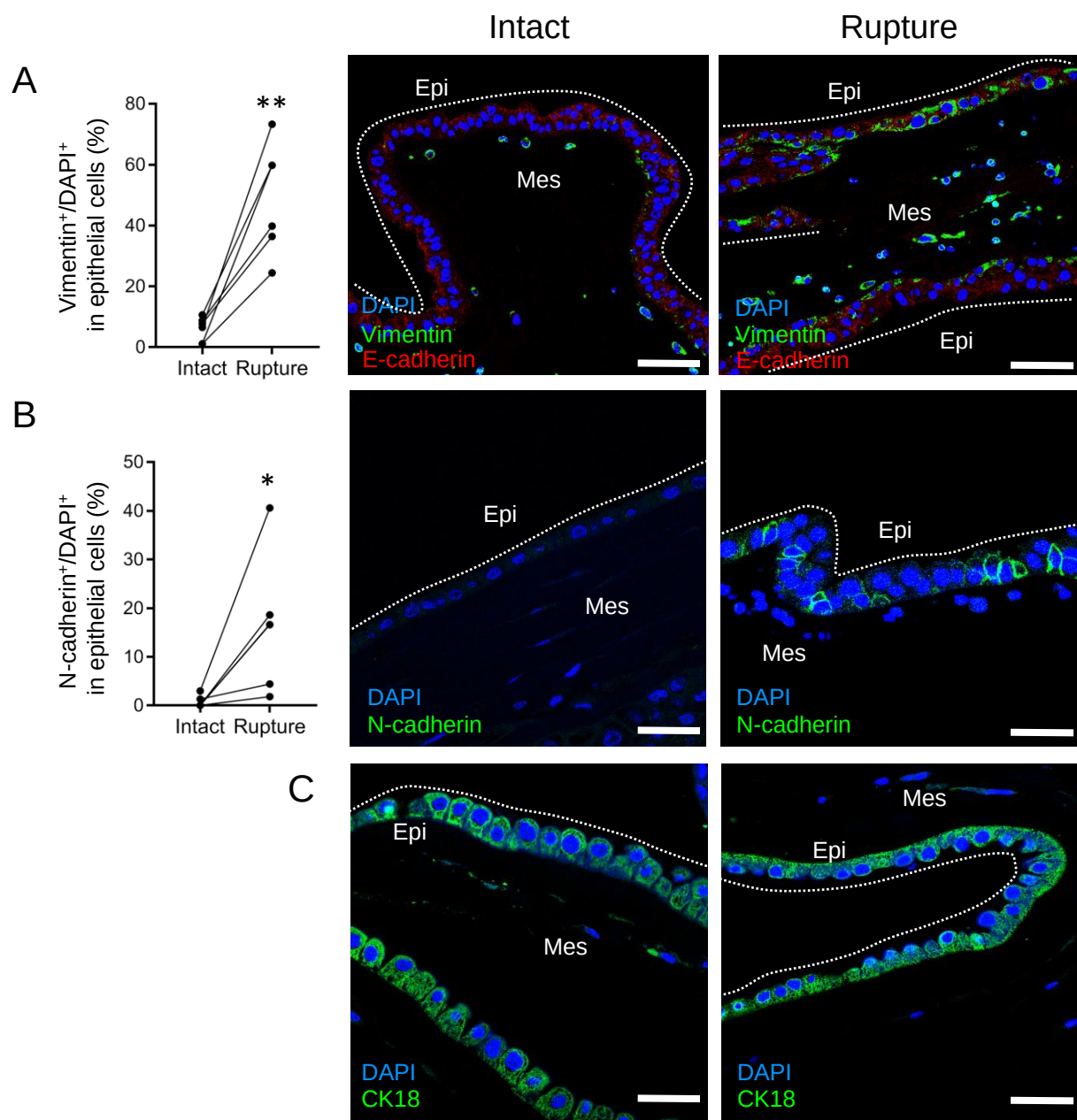


Fig. 3

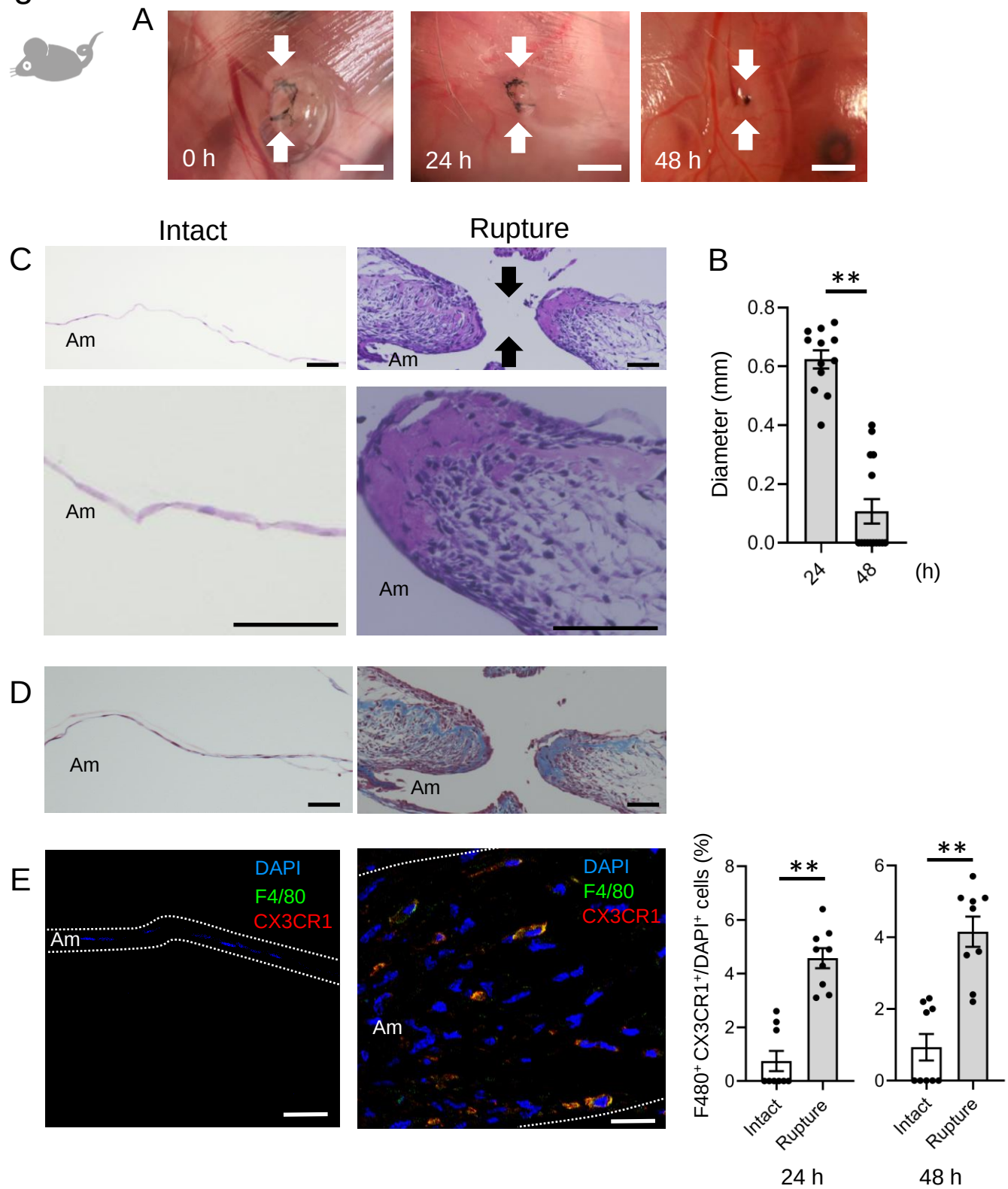


Fig. 4

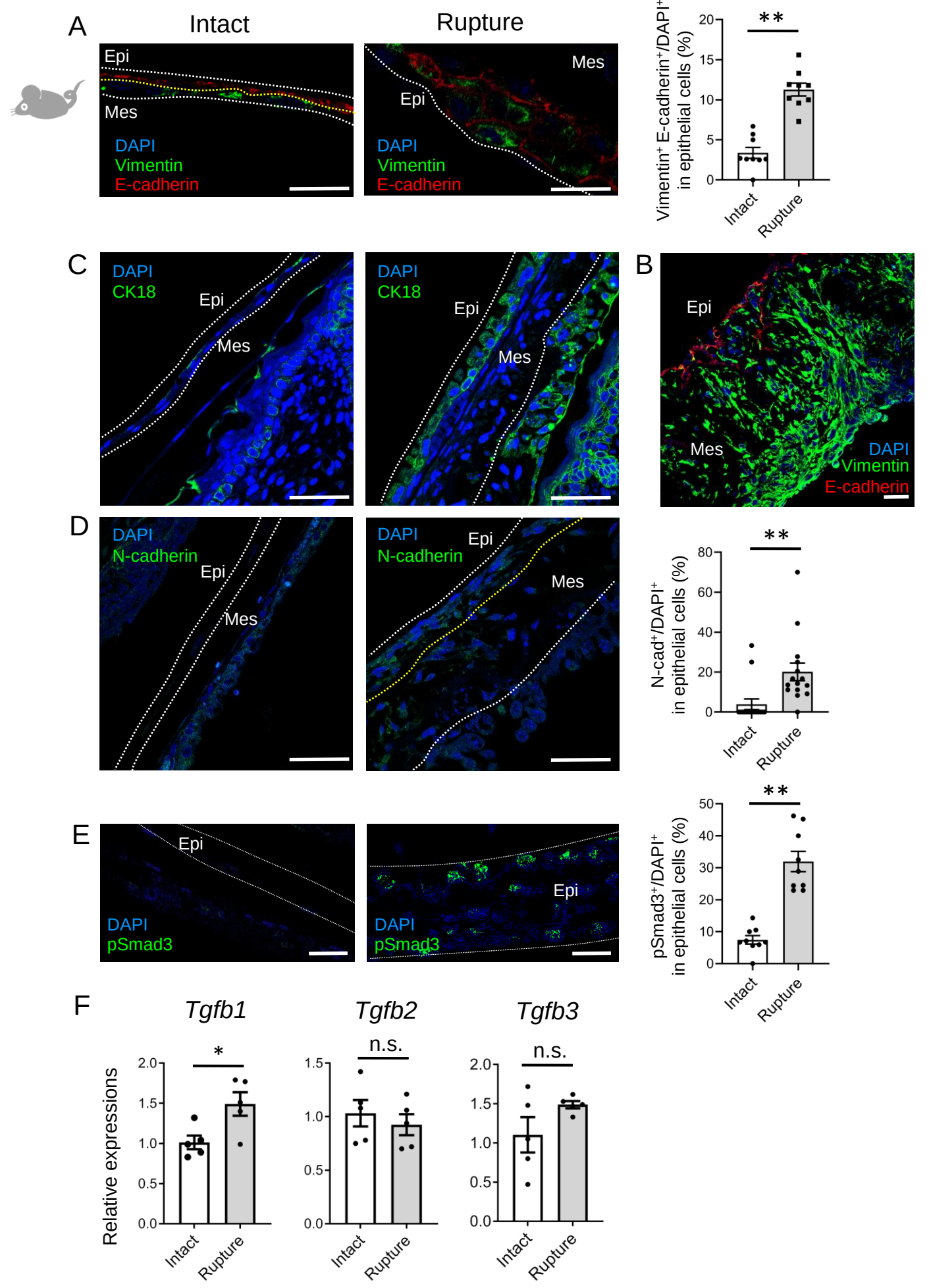


Fig. 5

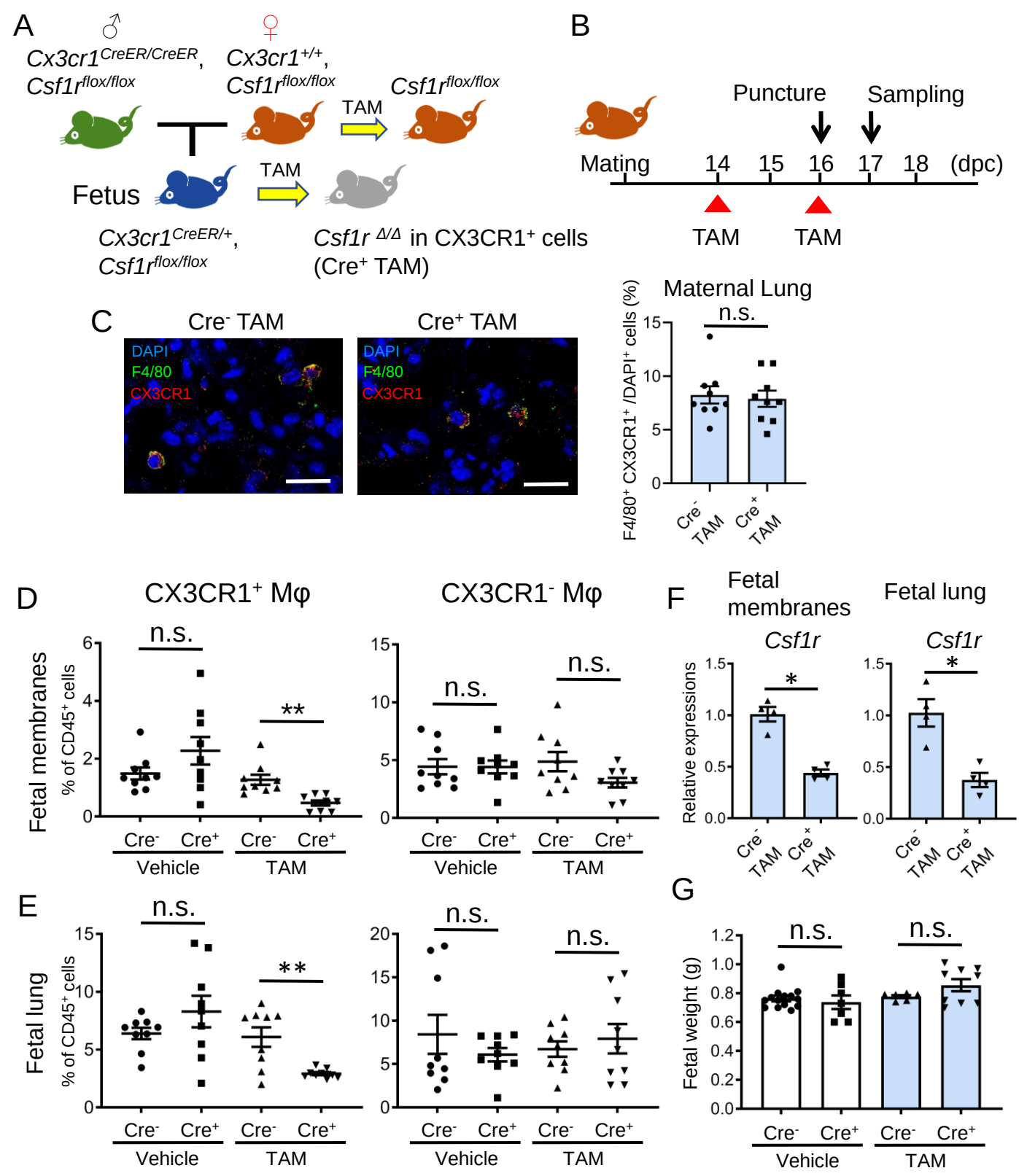
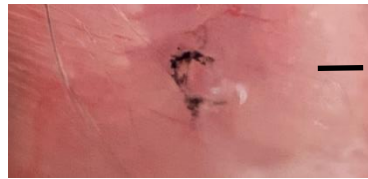


Fig. 6

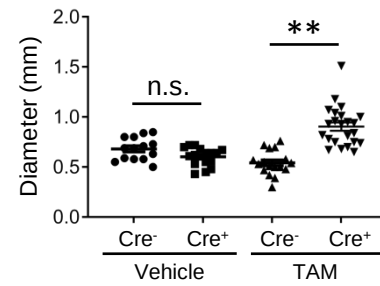
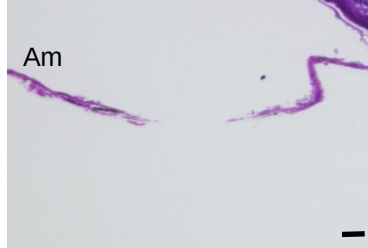
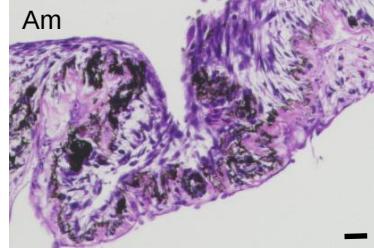
Cre⁻ TAM (Mφ⁺)

Cre⁺ TAM (Mφ⁻)

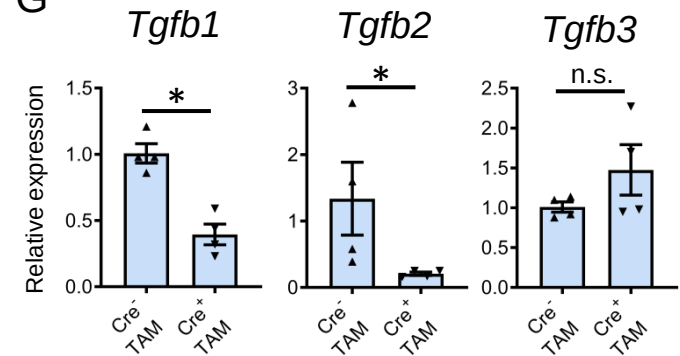
A



B

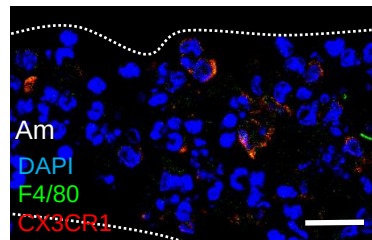


G

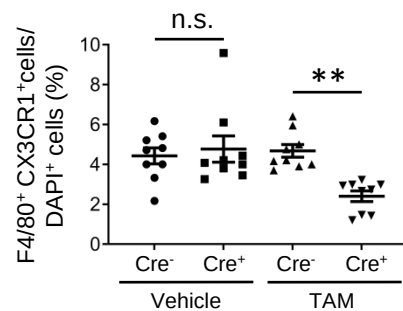
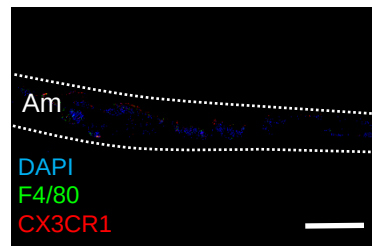


C

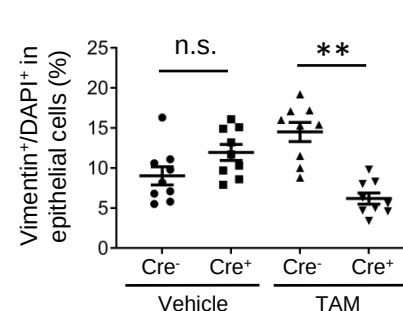
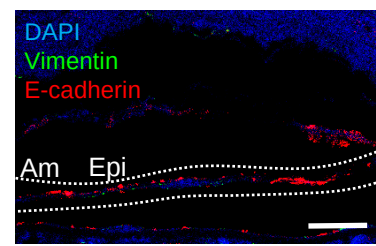
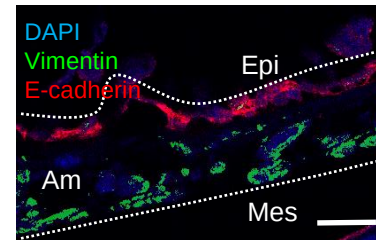
Cre⁻ TAM (Mφ⁺)



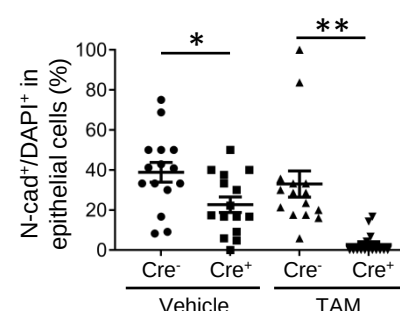
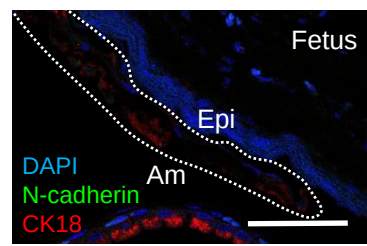
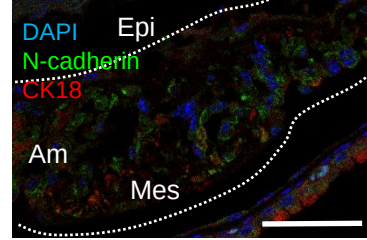
Cre⁺ TAM (Mφ⁻)



D



E



F

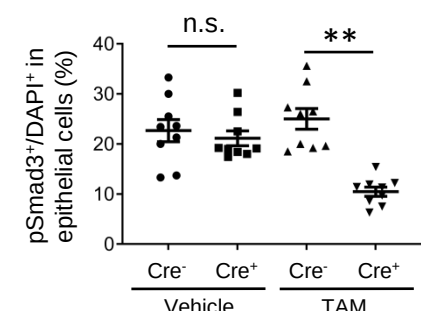
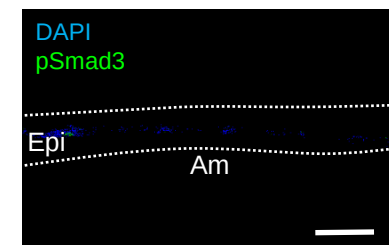
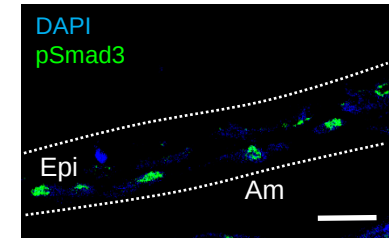


Fig. 7

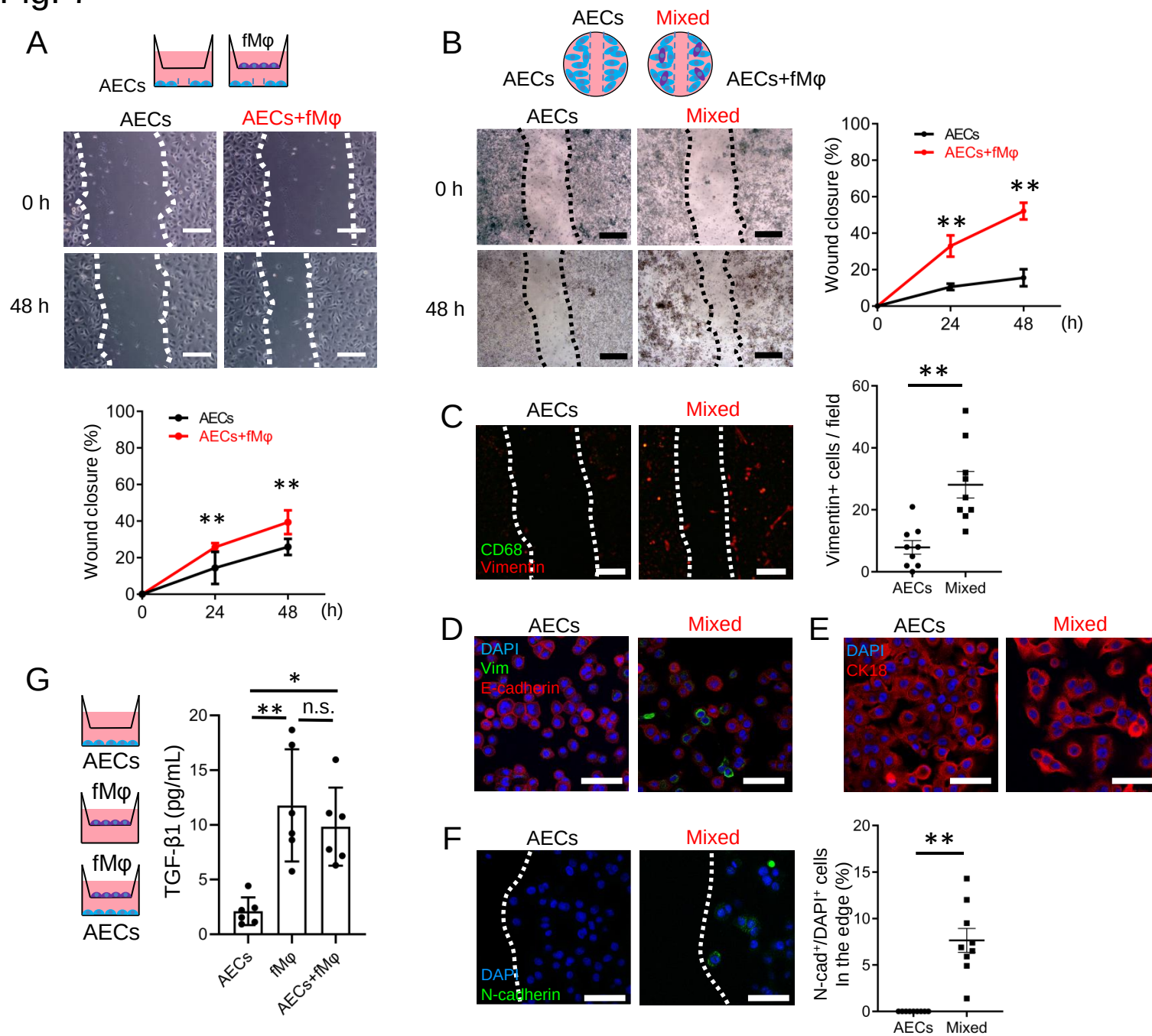


Fig. 8

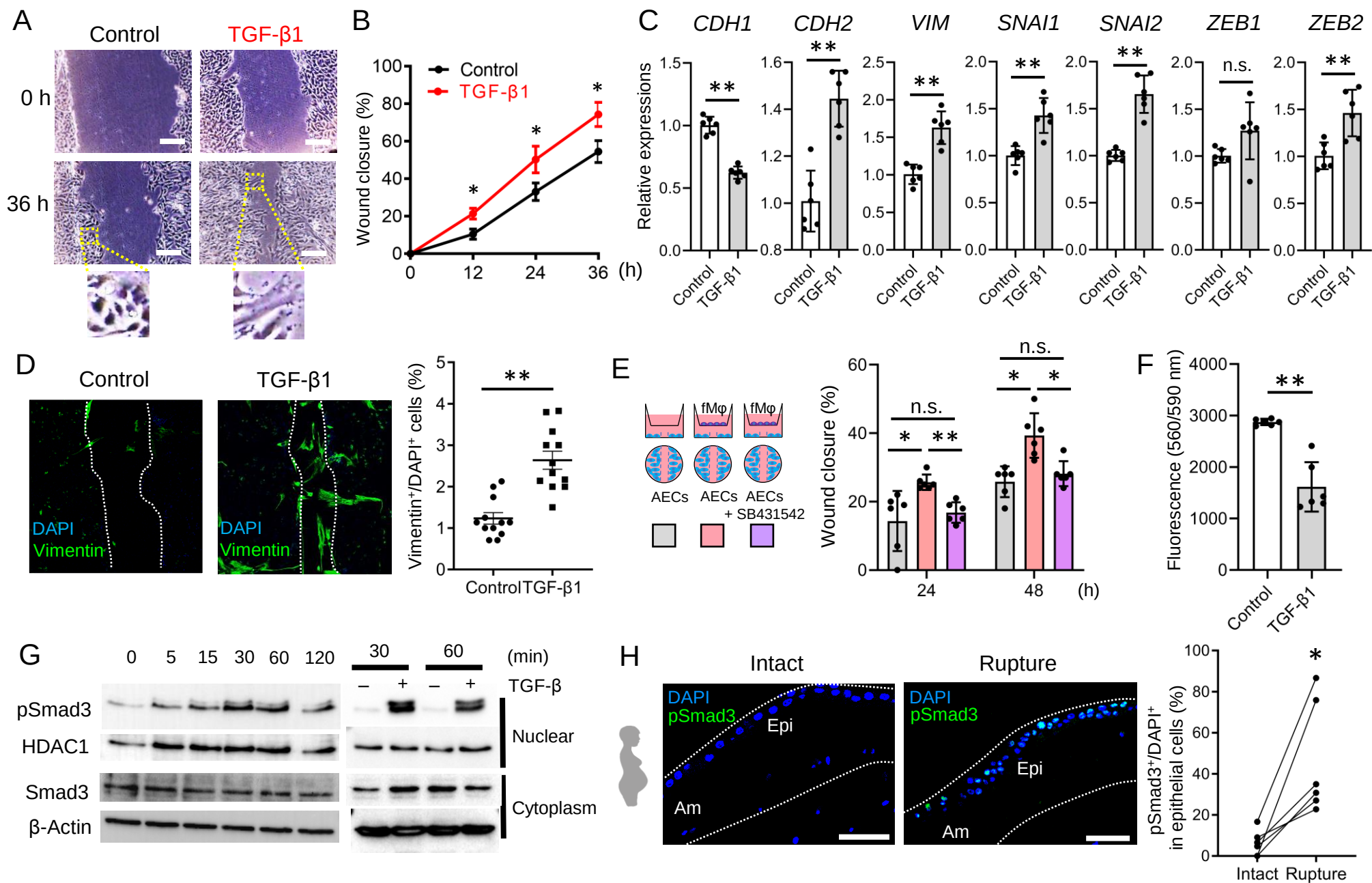


Fig. 8 continued

I

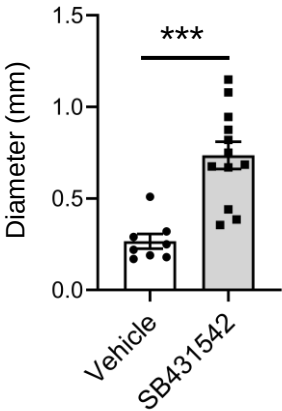
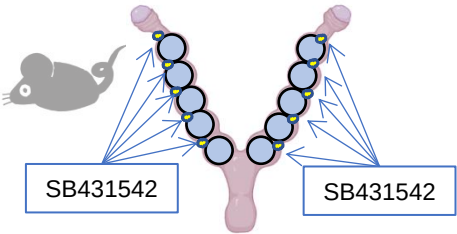


Fig. 9

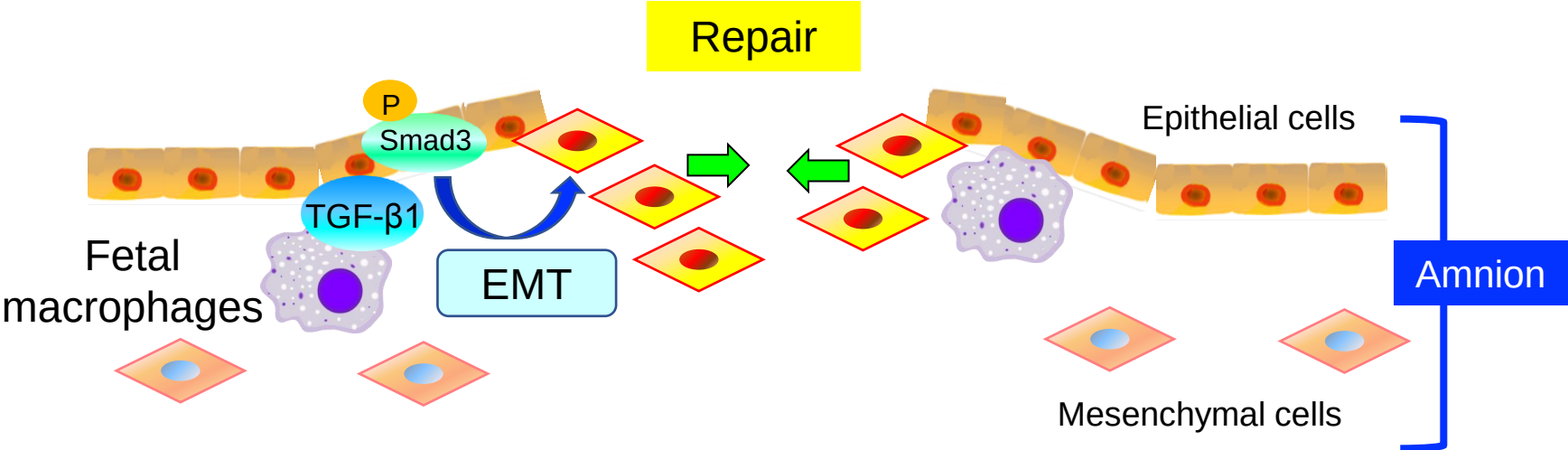
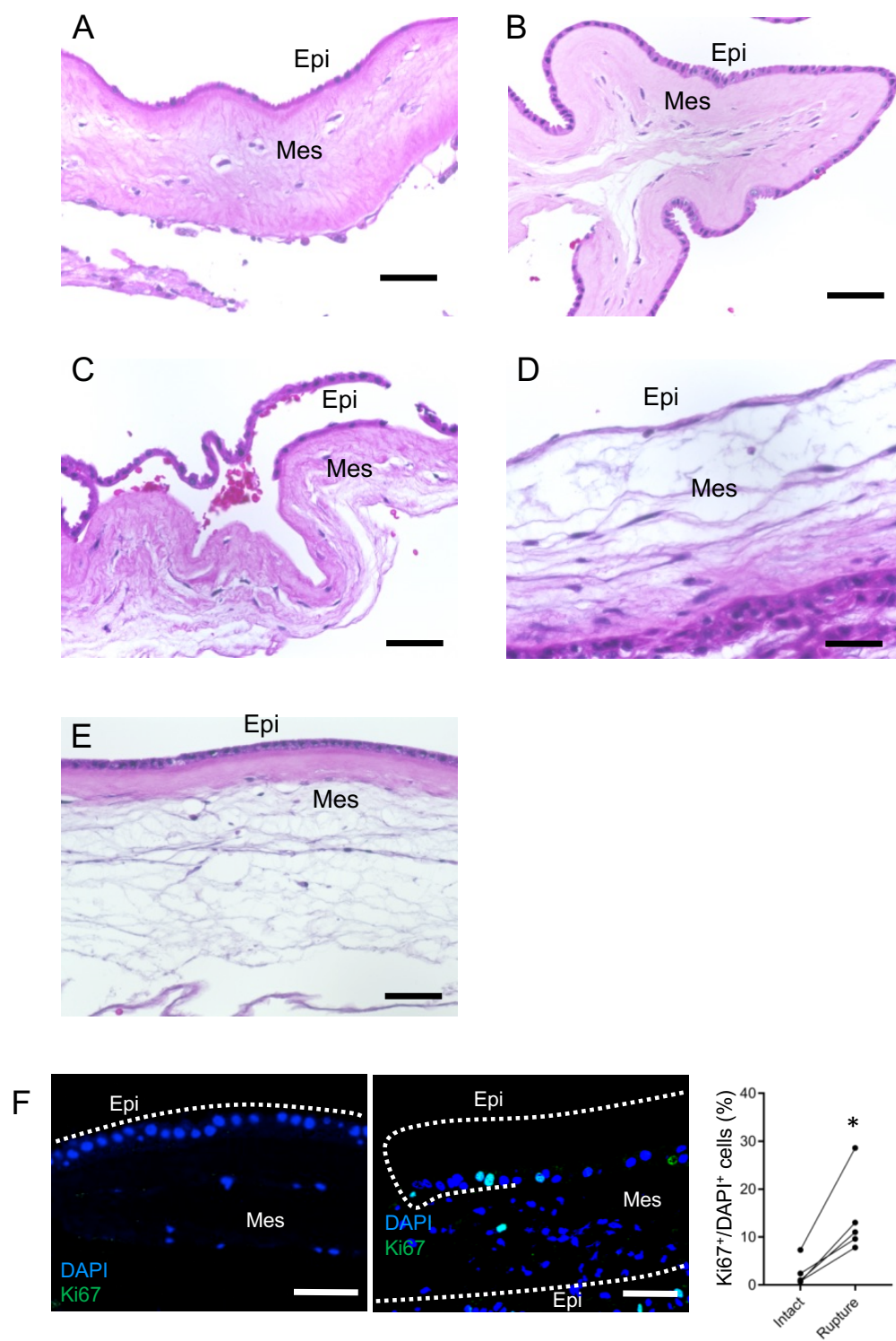


Fig. S1



1 **Fig. S1. Rupture site of human amnion. (A-E)** Hematoxylin-eosin (H&E) staining of the ruptured human
2 amnions used in this study. Case 2: GA 33 weeks (**A**), case 3: GA 29 weeks (**B**), case 4: GA 31 weeks (**C**), case 5,
3 GA 35 weeks (**D**), and case 6, GA 38 weeks (**D**). Bars, 50 μ m. (**F**) Representative immunofluorescence images for
4 Ki67 (green) and DAPI (blue) of the case described in Fig. 1A. Bars, 50 μ m. Graphs: the number of Ki67⁺ cells per
5 DAPI⁺ cells (n = 5 ROM cases). The edge is indicated by a yellow arrow. Epi: epithelial cell of amnion, Mes:
6 mesenchymal cells of amnion. * $p < 0.05$ using Wilcoxon matched-pairs signed rank test.

Fig. S2

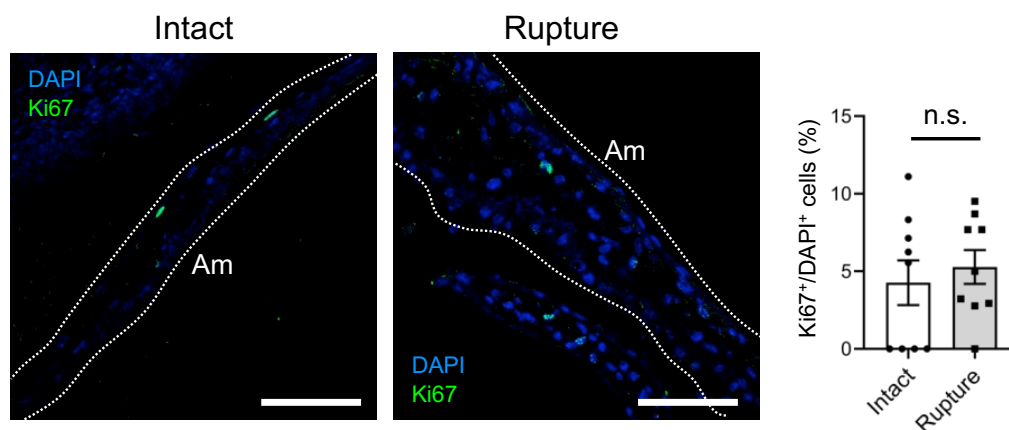


Fig. S2. Representative immunofluorescence images of Ki67 in the intact and ruptured murine amnion.

Graph: the number of Ki67⁺ cells per total DAPI⁺ cells in the field; n = 9 fields from 3 fetal membranes from 3 pregnant mice in each group. Bars, 50 μ m. Am: amnion. The graph shows the mean $\pm$ SEM of the values obtained. n.s., not significant. Two-tailed Mann-Whitney U test.

Fig. S3

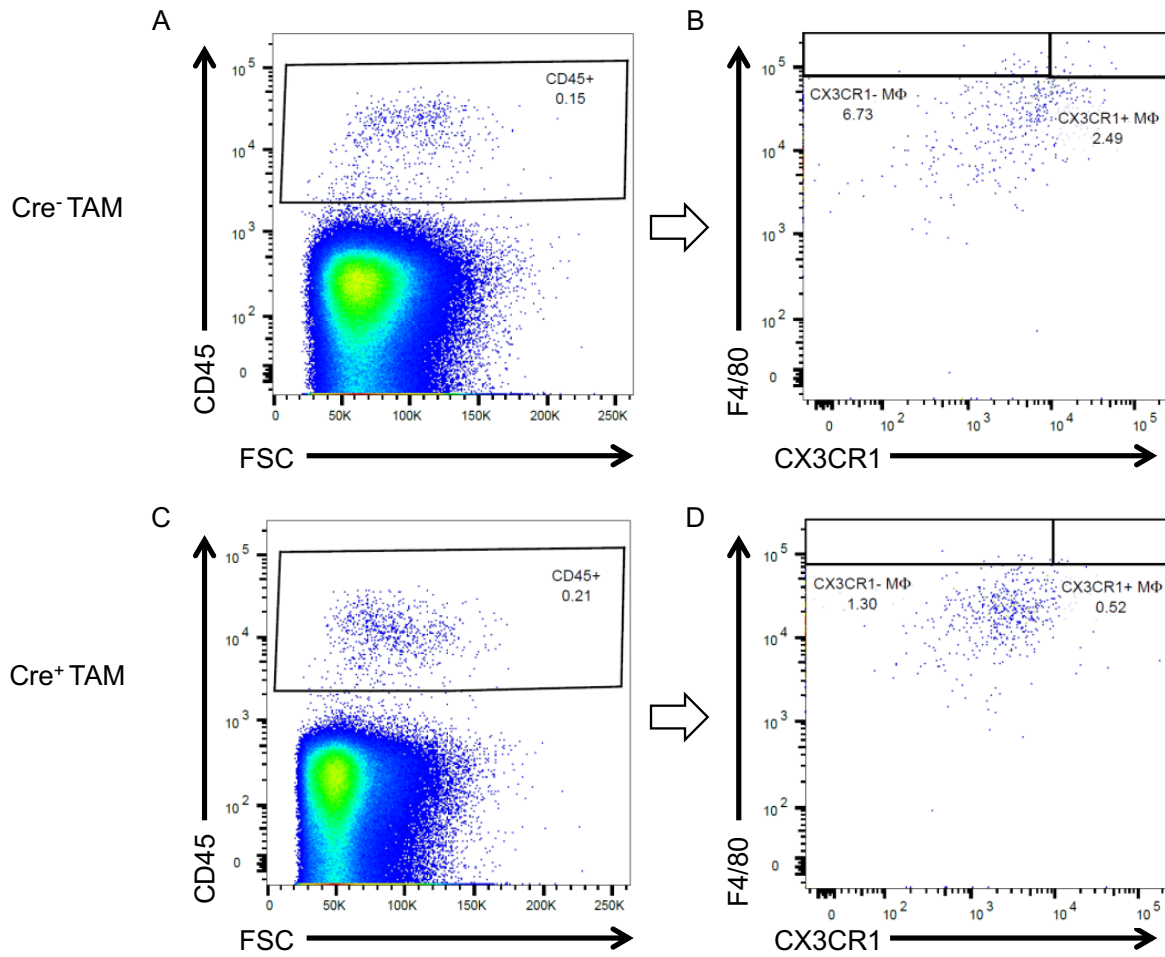


Fig. S3. Representative flow cytometry plots of fetal membrane samples from conditional knock-out mice.
A,B: Cre⁻ TAM fetuses. C,D: Cre⁺ TAM fetuses. A,C: leucocytes (CD45⁺ cells) in live cells. B,D: CX3CR1⁺ F4/80⁺ macrophages (CX3CR1⁺ MΦ) and CX3CR1⁻ F4/80⁺ macrophages (CX3CR1⁻ MΦ) in leukocytes.

Fig. S4

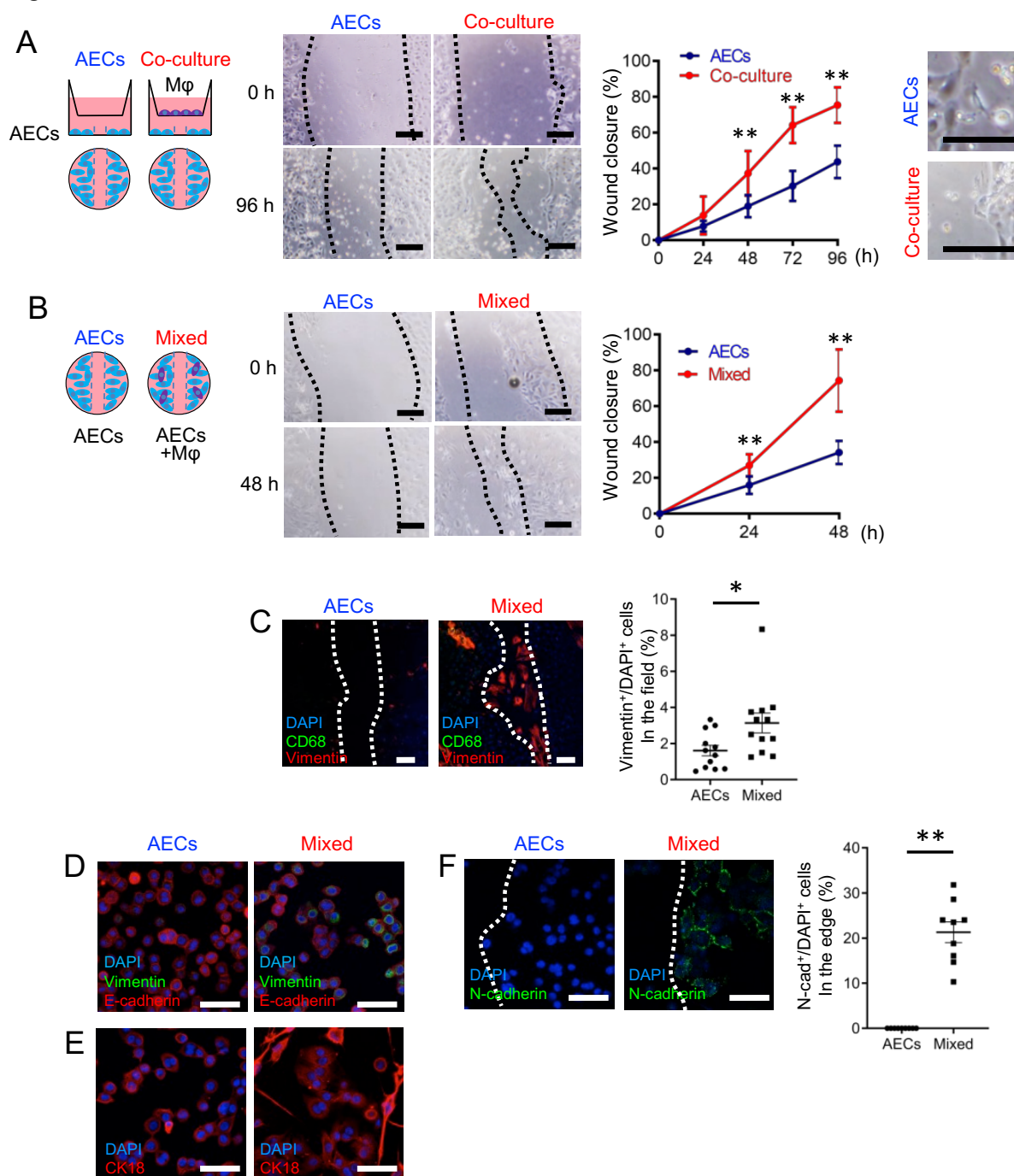


Fig. S4. Adult macrophages induce EMT in human AECs.

(A) Schematic showing how AECs were cultured alone or co-cultured with adult peritoneal macrophages (Mφ) in a separate compartment. Representative images from the wound healing scratch assay at 0 h and 96 h showing AECs cultured alone or co-cultured with Mφ (left). Bars, 200 μm. Dotted lines indicate the edge of the scratched area.

Graph: the percent coverage of scratched area as a function of time. AECs alone (blue line) or co-cultured with Mφ (red line). n = 6 biological replicates in each group, 3 independent experiments. Right, enlarged images to the left.

Bars, 200 μm. (B) Schematic showing how AECs were cultured alone or mixed with Mφ. Representative images

22 from the wound-healing scratch assay at 0 and 48 h showing AECs alone or mixed with Mφ (left). Bars, 200 μm.
23 Graph: the percent coverage of the scratched area as a function of time. n = 6 biological replicates in each group, 3
24 independent experiments. **(C-F)** Representative immunofluorescence images for CD68 and vimentin **(C)**, vimentin
25 and E-cadherin **(D)**, CK18 **(E)**, N-cadherin **(F)** and DAPI. in scratched AECs (left) or AECs mixed with Mφ (right).
26 Bars, 200 μm **(C)** and 50 μm **(D-F)**. Graphs: the number of vimentin⁺ cells per total DAPI⁺ cells in the field **(C)** or
27 N-cadherin⁺ cells per DAPI⁺ cells in the field **(F)**. n = 9-12 biological replicates per group, 3 independent
28 experiments. Graphs show means ± SD of the values obtained. n.s., not significant. * $p < 0.05$, ** $p < 0.01$ using two-
29 tailed Mann-Whitney U test.

Fig. S5

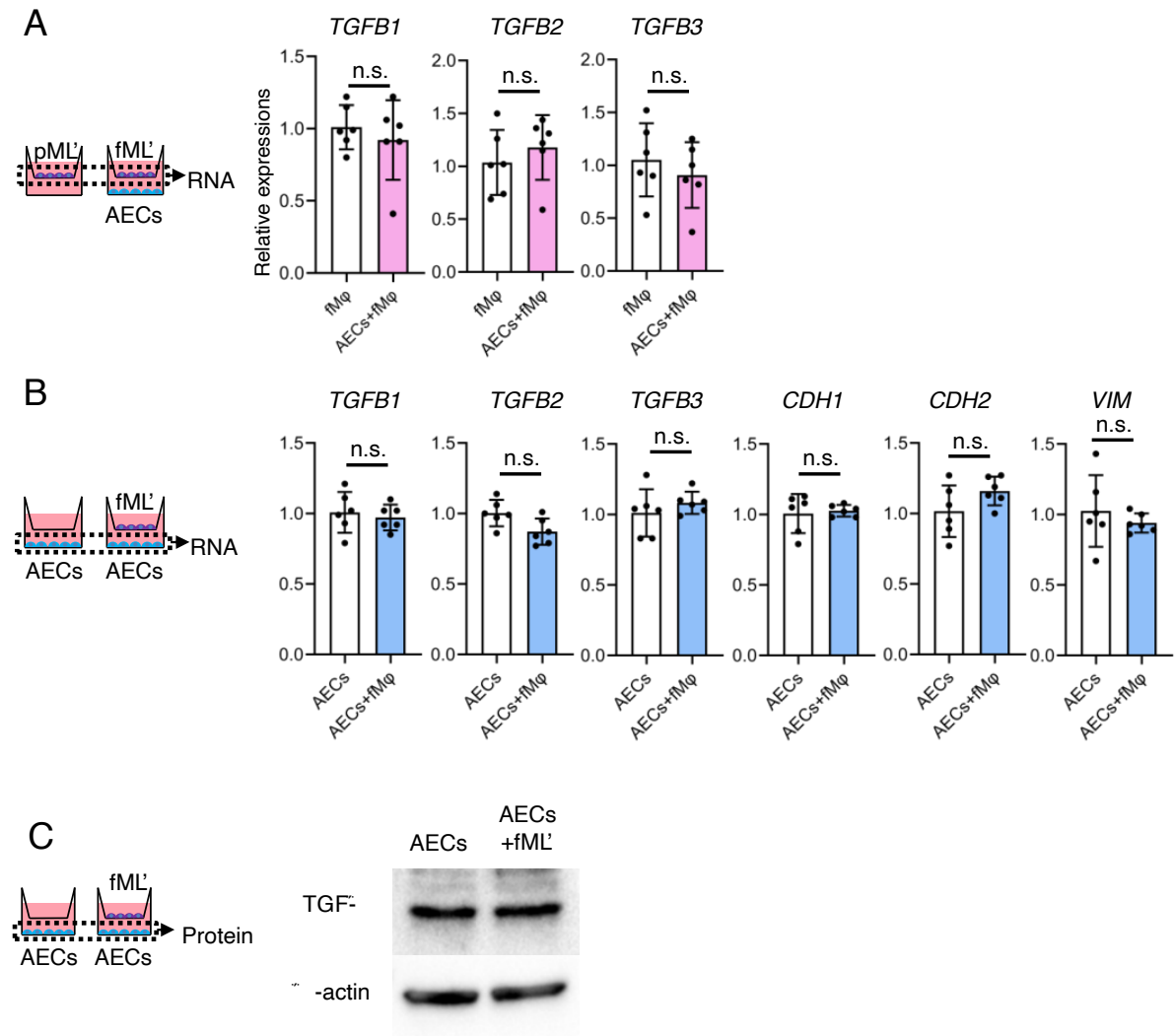


Fig. S5. Co-culture of human AECs with fetal macrophages.
(A, B, C) Schematic showing how fetal macrophages (fMφ) or AECs were cultured alone or the two cell types were co-cultured in separate compartment. (A, B) Quantitative PCR analysis of gene expression in AECs, fMφ or AECs+fMφ. n = 6 biological replicates in each group, 3 independent experiments. Graphs show means ± SD of the values obtained. n.s., not significant using two-tailed Mann-Whitney U test. (C) Representative immunoblots of TGF-β and β-actin in AECs or AECs+fMφ. Three independent experiments.

Fig. S6

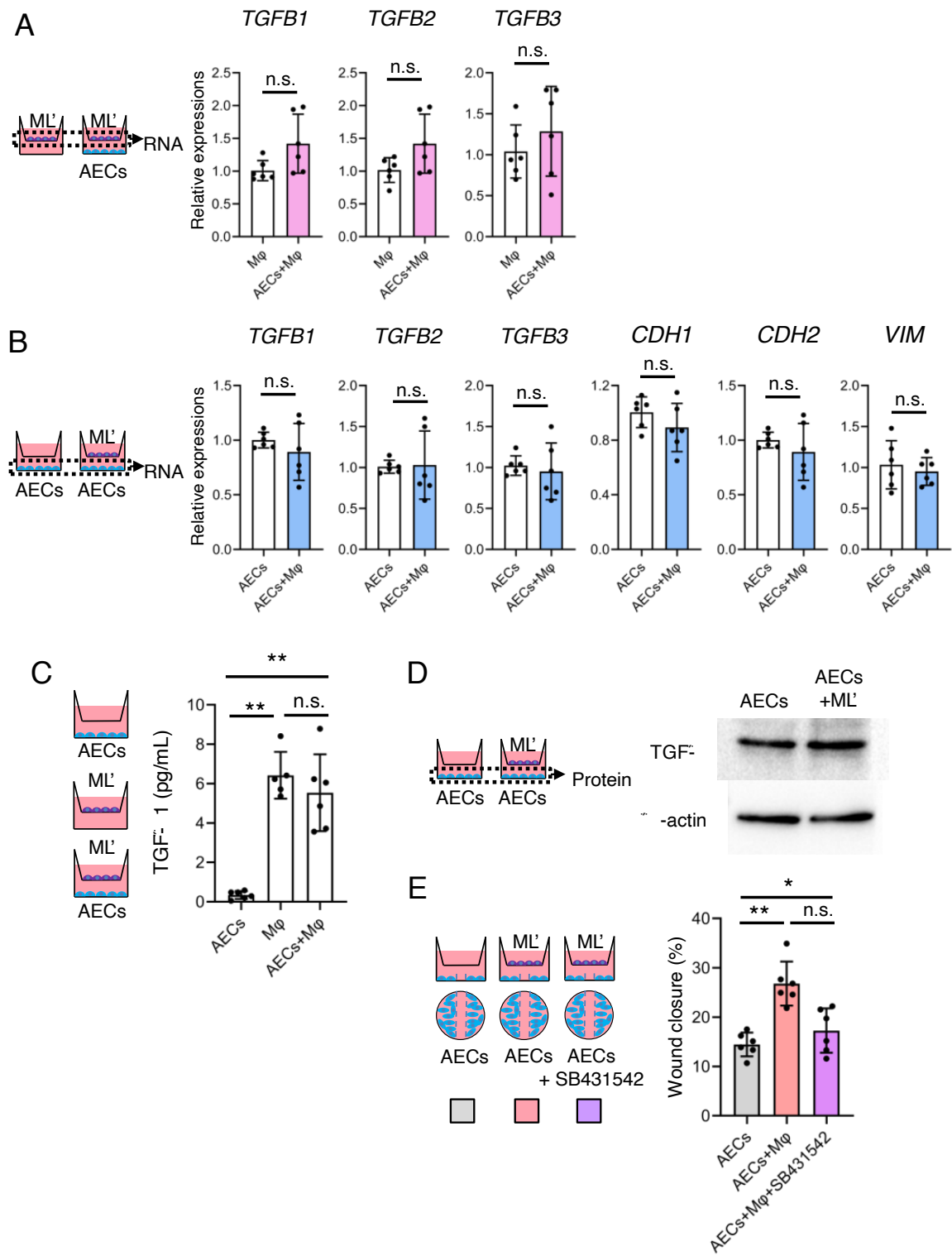


Fig. S6. Co-culture of human AECs with adult macrophages.

(A-C) Schematic showing how adult peritoneal macrophages (Mφ) or AECs were cultured alone or the two cell types were co-cultured in separate compartment. (A, B) Quantitative PCR analysis of EMT-related gene expression

in Mφ (**A**) or AECs (**B**). n = 6 biological replicates, 3 independent experiments. (**C**) TGF-β1 concentration in the conditioned medium from AECs cultured alone, Mφ cultured alone, or both cell types co-cultured together. n = 6 biological replicates, 3 independent experiments. Graphs show means ± SD of the values obtained. (**D**) Representative immunoblots of TGF-β and β-actin in AECs or AECs+Mφ. Three independent experiments. (**E**) Schematic of the migration assay using the TGF-β1 inhibitor SB431542 (10 μM). AECs were cultured alone, co-cultured with Mφ, or co-cultured with Mφ and SB431542. Graph: the percent closure of migration assay at 12 and 24 h. n = 6 biological replicates per group, 3 independent experiments. n.s., not significant, ***p* < 0.01. Two-tailed Mann-Whitney U test (**A**, **B**) or Kruskal-Wallis test followed by Dunn's multiple comparison test (**C**, **E**).

Table S1. Primary antibodies used in experiments.

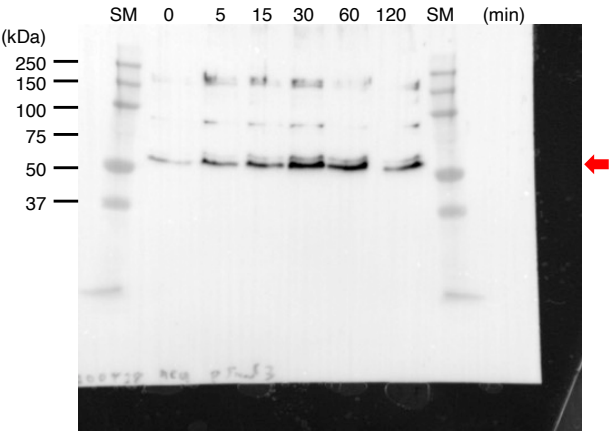
Immunohistochemistry					
Antibody	Company	No.	RRID	Antigen retrieval	Concentration
CD68	eBioscience	14-0688-80	AB_11151503	Sodium citrate	1/500
CD86	Cell signaling	91882	AB_2797422	Sodium citrate	1/200
CD206	BioLegend	321101	AB_571922	Sodium citrate	1/100
CX3CR1	eBioscience	14-6093-81	AB_467880	Sodium citrate	1/200
F4/80	eBioscience	14-4801	AB_467558	Proteinase K	1/50
Vimentin	Dako	M0725	AB_10013485	Sodium citrate	1/200
E-cadherin	Cell Signaling	14472	AB_2728770	Sodium citrate	1/100
N-cadherin (for human)	Cell Signaling	13116	AB_2687616	Tris/EDTA	1/125
N-cadherin (for mouse)	abcam	ab76011	AB_1310479	Sodium citrate	1/300
CK18 (for human)	Biolegend	628402	AB_439770	Tris/EDTA	1/100
CK18 (for mouse)	Proteintech	10830-1	AB_2133164	Sodium citrate	1/300
Ki67	eBioscience	14-5698-80	AB_10853185	Sodium citrate	1/100
phosho-Smad3	Abcam	ab52903	AB_882596	Tris/EDTA	1/200
Immunocytochemistry					
Antibody	Company	No.	RRID	Antigen retrieval	Concentration
CD68	eBioscience	14-0688-80	AB_11151503	N/A	1/500
Vimentin	Dako	M0725	AB_10013485	N/A	1/200
CK18	Proteintech	10830-1	AB_2133164	N/A	1/100
N-cadherin	Cell Signaling	13116	AB_2687616	N/A	1/200
Immunoblots					
Antibody	Company	No.	RRID	Antigen retrieval	Concentration
phosho-Smad3	Abcam	ab52903	AB_882596	N/A	1/2000
Smad3	Cell Signaling	9523	AB_2193182	N/A	1/1000
HDAC1	Proteintech	66085-1-Ig	AB_11232033	N/A	1/20000
TGF- β	R&D systems	MAB1835	AB_357931	N/A	1/500
β -actin	abcam	ab8227	AB_2305186	N/A	1/1000

RRID: Research Resource Identifiers

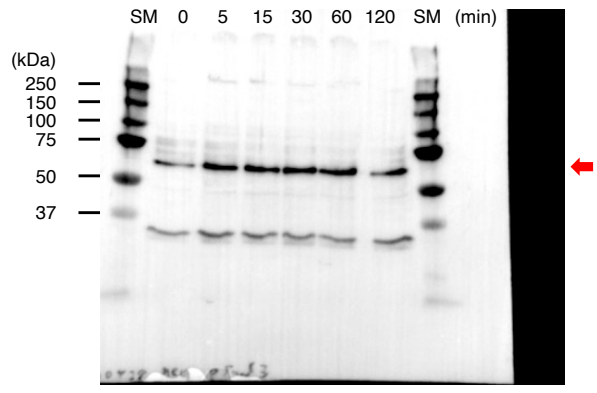
Table S2. Primer sequences used for qPCR

Mouse		
<i>Gene</i>	Forward	Reverse
<i>Tgfb1</i>	5'- AGCGCTCACTGCTCTTGTGA -3'	5'- GCTGATCCCGTTGATTCCA -3'
<i>Tgfb2</i>	5'- CCTTCGTGCCGTCTAATAATTACA -3'	5'- TGCCATCAATACCTGCAAATCT -3'
<i>Tgfb3</i>	5'- CCACCGTGTCTTCGACATTG -3'	5'- CGAAGTATCTGGAAGAGCTCAATTC -3'
<i>Csf1r</i>	5'- CAGCAATGATGTTGGCACA -3'	5'- TCAAGTTTAAGTAGGCACTCTCCA -3'
<i>B2m</i>	5'- CCGAGCCCAAGACCGTCTA -3'	5'- AACTGGATTGTGAATTAAGCAGGTTCA -3'
Human		
<i>TGFB1</i>	5'- ACTACTACGCCAAGGAGGTCAC -3'	5'- TGCTTGAACCTGTCATAGATTTTCG -3'
<i>TGFB2</i>	5'- CAAAGGTTACAATGCCAACTT -3'	5'- GCAGATGCTTCTGGATTATGG -3'
<i>TGFB3</i>	5'- GCTGGAGGAGATGCATGG -3'	5'- TTAGGGCAGACAGCCAGTTC -3'
<i>CDH1</i>	5'- TGGGCAGAGTGAATTTTGAAGA -3'	5'- TGAAACCGTAGAGGCCTTTTGA -3'
<i>CDH2</i>	5'- GGTGGAGGAGAAGAAGACCAG -3'	5'- GGCATCAGGCTCCACAGT -3'
<i>VIM</i>	5'-GGTTGATACCCACTCAAAAAGGA -3'	5'- TTCGTTGATAACCTGTCCATCTCTAG -3'
<i>SNAI1</i>	5'- ATGCCGCGCTCTTTCCT -3'	5'- GCTGGAAGGTAACTCTGGATTAGA -3'
<i>SNAI2</i>	5'- CCATTCCACGCCAGCTA -3'	5'- TCACTCGCCCCAAAGATGAG -3'
<i>ZEB1</i>	5'- GCACTTCCAGAATTAAAGCACTATGA -3'	5'- AAACAGAGGACTCAGGCTTCTCA -3'
<i>ZEB2</i>	5'- TTTTCCTGCCCTCTCTGTAGCT -3'	5'- GGTTAGCATTGGTGCTGATCTG -3'
<i>GAPDH</i>	5'- GGAGTCAACGGATTGGTCGTA -3'	5'- CAACAATATCCACTTTACCAGAGTTA -3'

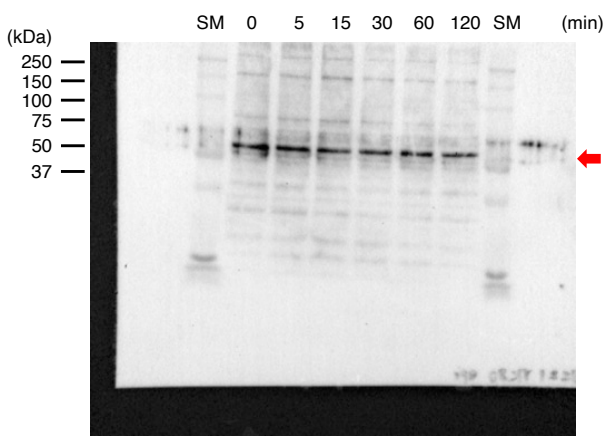
Epi TGF-beta treatment
IB: phospho Smad3 (EP823Y, 50 kDa)
Nuclear extract



Epi TGF-beta treatment
IB: HDAC1 (65 kDa)
Nuclear extract (reprobe)



Epi TGF-beta treatment
IB: Smad3 (50 kDa)
Cytoplasmic extract



Epi TGF-beta treatment
IB: beta-actin (42 kDa)
Cytoplasmic extract (reprobe)

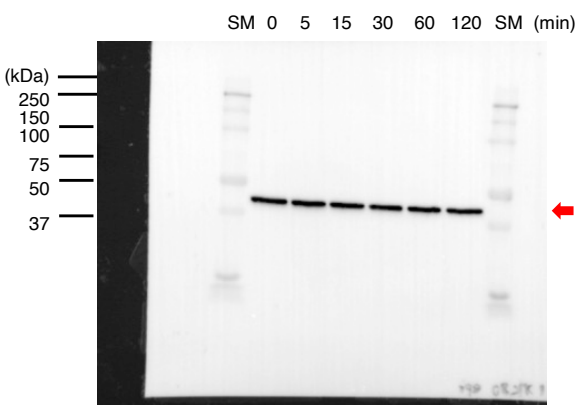


Fig. 8G, Uncropped immunoblots, biological replicate #1

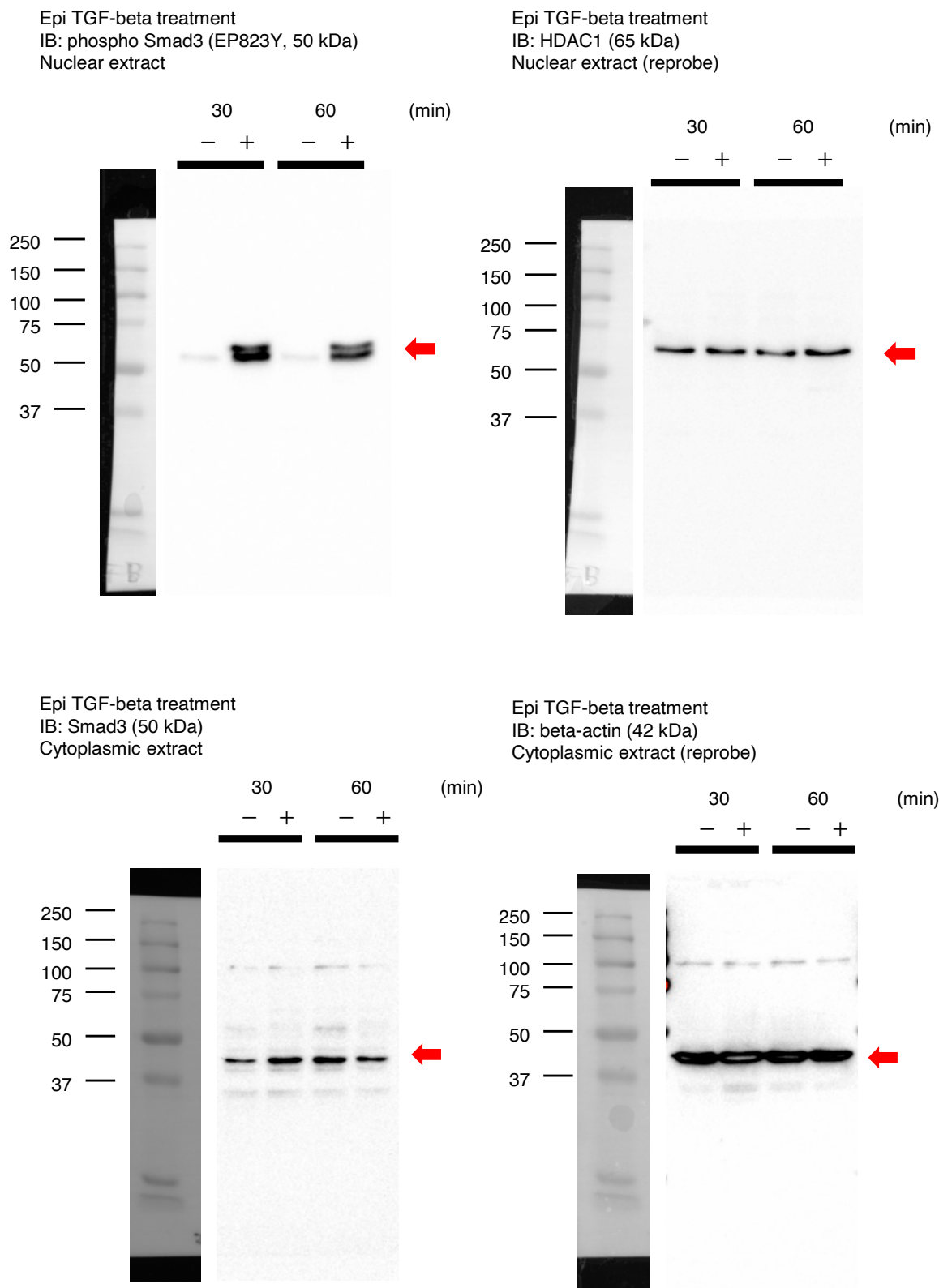
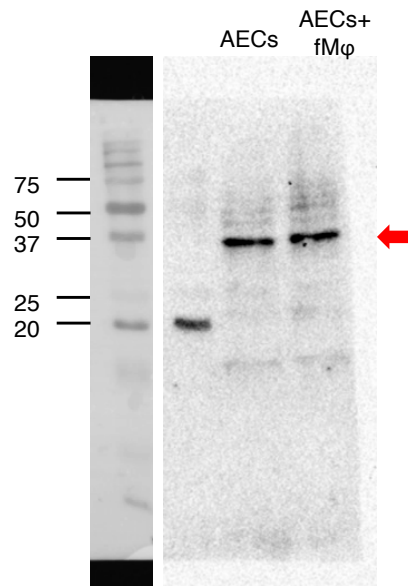


Fig. 8G, Uncropped immunoblots, biological replicate #1, continued

AECs + fetal (placental) M ϕ (fM ϕ) co-culture
 IB: TGFB (12, 25, 45-60 kDa)
 RIPA buffer



AECs + fetal (placental) M ϕ (fM ϕ) co-culture
 IB: beta-actin
 Reprobe

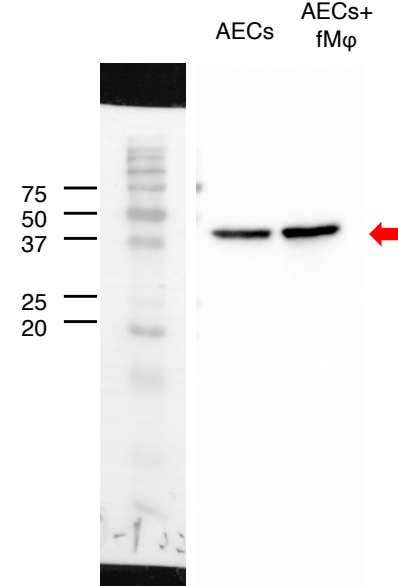
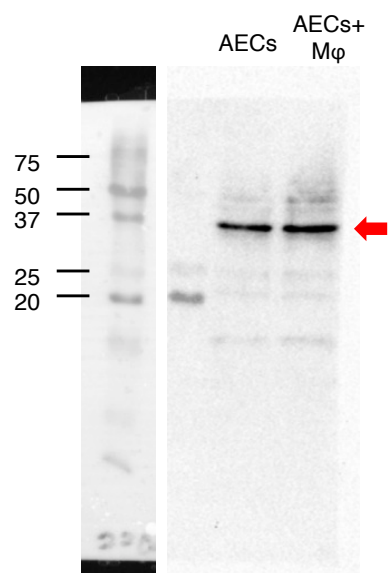
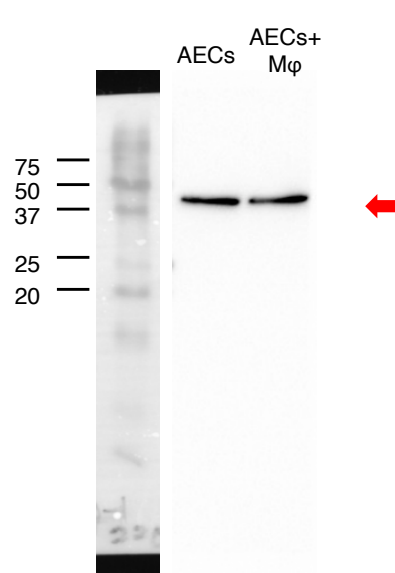


Fig. S5C, Uncropped immunoblots, biological replicate #1

AECs + adult (peritoneal) M ϕ co-culture
 IB: TGFB (12, 25, 45-60 kDa)
 RIPA buffer



AECs + adult (peritoneal) M ϕ co-culture
 IB: beta-actin
 Reprobe



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 68 **Fig. S6D, Uncropped immunoblots, biological replicate #1**

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