Sequential formation and resolution of multiple rosettes drives embryo remodeling after implantation

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Summary

The dramatic morphogenetic remodeling of embryo architecture after implantation culminates in pro-amniotic cavity formation. Despite its key importance, how this transformation occurs remains unknown. Here, we apply high-resolution imaging of embryos developing \textit{in vivo} and \textit{in vitro}, spatial RNA sequencing and 3D trophoblast-stem-cell models to determine the sequence and mechanisms of these remodeling events. We show that cavitation of the embryonic tissue is followed by folding of extra-embryonic tissue to mediate formation of a second, extra-embryonic cavity. Concomitantly, at the boundary between embryonic and extra-embryonic tissues, a hybrid 3D rosette forms. Resolution of this rosette enables the embryonic cavity to invade the extra-embryonic tissue. Subsequently, $\beta_1$-integrin signalling mediates formation of multiple extra-embryonic 3D rosettes. Podocalyxin exocytosis leads to their polarized resolution permitting extension of embryonic and extra-embryonic cavities and their fusion into a unified pro-amniotic cavity. These morphogenetic transformations of embryogenesis bring a novel mechanism for lumen expansion and fusion.

\textbf{Keywords:} mammalian embryo, post-implantation morphogenesis, 3D rosette, lumen formation
Introduction

Embryogenesis involves a gradual increase in complexity of embryo architecture as cells and tissues take shape. These events require intricate coordination and in mammals involve close cooperation between embryonic and extra-embryonic tissues that become established by the time of implantation\textsuperscript{1-4}. The embryonic tissue, the epiblast, will give rise to the new organism, while one of the two extra-embryonic tissues, the trophectoderm, will generate the extra-embryonic ectoderm giving rise to the placenta and the other, the primitive endoderm, will generate the visceral endoderm and finally the yolk sac. The critical interaction between these tissues during mouse implantation development enables the transformation of the blastocyst into the egg cylinder, a more complex structure with an entirely different architecture.

The major morphogenetic event in the blastocyst-to-egg cylinder transition involves formation of the pro-amniotic cavity that spans the whole length of the egg cylinder. We have shown that this morphogenesis is initiated by polarization and lumenogenesis of the embryonic compartment that is triggered by β1-integrin signalling\textsuperscript{5,6} and coordinated with a transition in stem cell potential\textsuperscript{5,6}. However, how the lumen becomes established in the trophectoderm-derived extra-embryonic compartment and how then the embryonic and extra-embryonic lumens unify to form single cavity have remained unknown. Here, we use mouse embryos developing \textit{in vivo} and \textit{in vitro} together with 3D stem cell models, high resolution time-lapse microscopy and spatio-temporal transcriptome analyses to determine the sequence of events and mechanisms underlying coordination between the embryonic and extra-embryonic tissues in driving embryo remodeling and pro-amniotic cavity formation.

Results

Morphogenetic steps of pro-amniotic cavity formation

To understand how the pro-amniotic cavity forms, we first sought to determine the sequence of morphogenetic events involved. Based on detailed observations of 142 embryos freshly recovered during implantation stages, we could distinguish five distinct stages of global tissue rearrangements culminating in pro-amniotic cavity formation (Fig. 1a-e). During stage I (E4.75-5.0 embryos), epiblast cells became polarized into a rosette-like structure with a lumen opening at its centre, as previously described\textsuperscript{5}, while trophectoderm cells proliferated with the most proximal cells undergoing apical constriction. In stage II (E5.25-5.5 embryos), a thin elongated cavity formed at the proximal end of the extra-embryonic ectoderm as a result of tissue folding (Fig. 1f-h; Fig. S1, Movie 1-3). During stage III (E5.5 embryos), the
epiblast rosette re-organized into a cup-shaped epithelium while extra-embryonic ectoderm cells at the embryonic/extra-embryonic boundary became polarized with their apical sides flanking the epiblast cavity. At the same time, the apico-basal polarity axis of the epiblast at the embryonic/extra-embryonic boundary changed from parallel to perpendicular, relative to the proximo-distal axis of the embryo (Fig. 1b,e). During stage IV (E5.5-E5.75 embryos), the embryonic cavity extended into the extra-embryonic compartment (Fig. 1b). Finally, during stage V (E5.75 embryos), the two cavities fused into a unified single cavity spanning the whole embryo. These observations allowed us to determine the sequence of cellular re-organization that remodels the embryonic and extra-embryonic tissues leading to pro-amniotic cavity formation.

**Extra-cellular matrix is required for morphogenesis of extra-embryonic compartment**

As extra-cellular matrix (ECM) signalling directs the formation of a cellular rosette that undergoes lumenogenesis in the embryonic compartment, we wondered whether a similar mechanism operates in the extra-embryonic compartment. High resolution analysis of tissue remodeling on the cellular level revealed that immediately upon implantation, the extra-embryonic compartment is comprised of two cell populations: one on the outside in direct contact with the basement membrane (BM), and a second on the inside (n=35 embryos, Fig. 2a). While the outside cells were apico-basally polarized, as revealed by the distribution of Golgi apparatus and cell shape, the inside cells were not (Fig. 2a-c). Since laminin is a ligand for integrin receptors, we hypothesized that such a polarization mechanism could act via the ECM surrounding the extra-embryonic compartment. Analysis of the activation status of β1-integrin, the main ECM signalling mediator on the basal side of the outside extra-embryonic cells (Fig. S2a), supported this hypothesis.

To determine whether the ECM is sufficient to provide the signalling cues required for polarization of the extra-embryonic ectoderm, we established a trophoblast stem cell (TSC) 3D model to mimic the development of the extra-embryonic compartment in vitro. This entailed the suspension of small clumps of TSCs in 3D Matrigel (as ECM substitute). We found that provision of ECM components led TSCs to arrange into spherical aggregates resembling the extra-embryonic compartment (n=20, Fig. 2d). Whereas the TSCs in contact with the ECM acquired columnar polarized morphology, TSCs not in contact with the ECM retained an apolar character (Fig. 2d-e), supporting a role for the ECM in the polarization of
extra-embryonic cells. TSCs clumps cultured in the absence of the ECM failed to polarize confirming that the ECM is necessary for TSCs polarization (Fig. S2b).

To determine whether provision of the ECM is indeed required for polarization of extra-embryonic ectoderm in vivo, we disrupted the ECM by treating embryos with collagenase IV (COLIV)⁹. The COLIV treatment led to BM disruption and defective β1-integrin activation (Fig. S2c-d): whereas outside cells in control embryos polarized, the outside cells of COLIV-treated embryos did not (n=12 and 13 respectively; Fig. 2f-g; Fig. S2e). These results indicate that ECM-mediated signalling is necessary for extra-embryonic ectoderm organization.

**ECM/β1-integrin signalling is required for extra-embryonic compartment cavitation**

As β1-integrin signalling plays a central role in cell polarization in many different systems⁵,¹⁰, we therefore hypothesized that it might be required for polarization of the extra-embryonic compartment. Since β1-integrin knock-out embryos die during the implantation period¹¹, we tested our hypothesis using the TSC 3D system and a β1-integrin blocking antibody¹². TSCs cultured in the presence of the β1-integrin blocking antibody became disorganized and apolar, in contrast to the organized and polarized control aggregates (n=20 for each group, Fig. 2h-j; Fig. S2f). Therefore, blocking β1-integrin function caused failure of TSC polarization that resembled the effects of ECM disruption and loss of β1-integrin activity upon extra-embryonic ectoderm polarization.

Since the above results indicated that extra-embryonic ectoderm polarizes in response to an ECM/β1-integrin-dependent mechanism, we next assessed whether ECM/β1-integrin-mediated polarization is essential for pro-amniotic cavity morphogenesis. To this end we recovered embryos just after implantation and cultured them for 24 hrs in the absence or presence of COLIV. Whereas in the great majority of cultured control embryos the embryonic and extra-embryonic cavities fused, this process failed in most COLIV-treated embryos (Fig. 2k-l). Importantly, COLIV-treated embryos in which the formation of the pro-amniotic cavity was unaffected (3/27 embryos) had a residual BM (Fig. 2k right panel). These results indicate that ECM/β1-integrin-mediated polarization of the extra-embryonic ectoderm is essential for pro-amniotic cavity morphogenesis.

**ECM/β1-integrin signalling drives the formation of extra-embryonic rosettes**

To determine the cellular dynamics as the extra-embryonic compartment forms, we analyzed 82 freshly collected embryos prior to the fusion of the cavities. We found that in contrast to
the embryonic compartment, the extra-embryonic compartment of nearly all E5.5 embryos examined (93%, n=82) had multiple rosette structures (Fig. 3a; Fig. S3a). These extra-embryonic rosettes comprised 6 to 10 cells (n=60 rosettes, 40 E5.5 embryos) and had all the features of epithelial rosettes described thus far\textsuperscript{13,14}: the apical localization of polarity markers (aPKC, Par6, ZO-1) and actomyosin, indicated that extra-embryonic ectoderm cells were apically polarized towards the rosette’s centre (Fig. 3b). Strikingly, the number of extra-embryonic rosettes depended on the developmental stage: they were present more frequently in stages II and III; their frequency was significantly reduced in stage IV and were completely absent in stage V (so after cavities fusion) (Fig. 3c).

The majority of epithelial rosettes described so far are 2D structures forming within single-layered epithelia\textsuperscript{15}. 3D cell segmentation analyses revealed that extra-embryonic rosettes are 3D structures (Fig. 3d-e; Movie 4). However, in contrast to other 3D rosettes that participate in lumen formation\textsuperscript{5,16}, the extra-embryonic rosettes do not form lumens (n=60 rosettes, 40 E5.5 embryos) and, unusually, two adjacent rosettes could share cells that are bipolar contributing to both a rosette and the cavity (Fig. 3d-f; Movie 5).

To gain insight into the role of rosettes during extra-embryonic morphogenesis, we examined their formation. Analyses of 142 embryos revealed that as many as 95% of the rosettes only contacted the BM on one side (with their outer cells) and had a second group of inner cells that, although polarized with their apical side towards the rosette’s centre, were not in contact with the BM (Fig. S3b-d). This led us to hypothesize that inner cell polarization might be dependent upon outer cells becoming polarized through ECM/\(\beta_1\)-integrin signalling. To test this hypothesis, we analyzed the organization of rosettes in COLIV-treated embryos in which polarization of outside cells was prevented. In contrast to all control embryos, rosette formation was defective in the presence of COLIV (n=12 and n=12 control and experimental embryos respectively) (Fig. 3g-h). Together, these results suggest that ECM-\(\beta_1\)-integrin signalling is required for polarization of outside cells as well as subsequent polarization of inside cells and rosette formation (Fig. 3i).

**Resolution of extra-embryonic rosettes drives pro-amniotic cavity formation**

The defective pro-amniotic cavity formation seen in the absence of extra-embryonic rosettes suggested an involvement of these rosettes in cavity formation. To address this possibility, we analyzed the timing of events leading to the appearance of rosettes in relation to the five stages of pro-amniotic cavity formation we observed here (Fig. 1a-b). We found that just...
before the re-organization of the epiblast from a sphere to a cup-shaped epithelium, a rosette consisting of embryonic and extra-embryonic cells was established at the boundary between the compartments (Fig. 4a). The formation of this hybrid rosette was preceded by the breakdown of the BM separating the two compartments at stage I (E4.75; n=20 embryos, Fig. 4b). Importantly, resolution of this hybrid rosette was concomitant with epiblast remodeling from a sphere to a cup-shaped epithelium (E5.5, n=10 embryos, Fig. 4c).

To establish the exact sequence of these events, we filmed the development of embryos expressing LifeAct-GFP transgene\textsuperscript{17}. This revealed a strong apical enrichment of actin at the centre of the hybrid rosette. This actin enrichment was lost just before resolution of the hybrid rosette, allowing the cavity to progress into the extra-embryonic compartment (Fig. 4d; Fig. S4, Movie 6,7). We confirmed that epiblast cells contributing to a hybrid rosette re-oriented their polarity so that a tract of cells with common polarization connected the centre of the rosette with the cavity (Fig. 4e). To determine if loss of cell-cell adhesion along this track drives cavity extension, we laser ablated epiblast cells at the adhesion sites connecting the epiblast cavity with the centre of the hybrid rosette. This laser ablation led to hybrid rosette resolution and cavity progression (Fig. 4f; Movie 8), confirming that loss of adhesion at specific sites of epiblast cells drives the cavity progression.

After hybrid rosette resolution, a new rosette became established near the embryonic/extra-embryonic boundary in proximity to the expanding embryonic cavity (Fig. 4d). This rosette comprised only extra-embryonic cells and was detected only in stage II and III embryos (n=20 embryos; Fig. S5a). The main morphological change during the transition from stage III to IV was the extension of the embryonic cavity. We found that at stage III, embryos typically displayed a tract of the polarized apical parts of cells extending from the tip of the embryonic cavity to the centre of an adjacent extra-embryonic rosette (Fig. 5a; Movie 9). When embryos reached stage IV, extra-embryonic cells facing the embryonic cavity acquired an open rosette conformation (Fig. 5b; Fig. S5b), suggesting that resolution of the rosettes drives the extension of the cavity during the transition from stage III to IV.

To visualize the dynamics of cavity extension, we filmed the development of embryos expressing membrane-Tomato\textsuperscript{18} or LifeAct-GFP. This revealed that the resolution of a rosette near the embryonic/extra-embryonic boundary preceded the extension of the epiblast cavity (Fig. 5c; Fig. S5c; Movie 10-11). Specifically, rosette cells lost adhesion at cell interfaces connecting the epiblast cavity with the centre of the rosette (blue and purple cells Fig. 5c,
magenta and cyan cells Fig. S5c) in agreement with our observations of embryos developing in vivo.

Finally, we found that the centres of rosettes appearing near the extra-embryonic cavity were linked with the tip of the extra-embryonic cavity through polarized tracts, priming the cavity’s expansion during stage IV (Fig. 5d; Fig. S5d-e; Movie 12). These observations suggest that progressive expansion of embryonic and extra-embryonic cavities upon resolution of rosettes results in their fusion (Fig. S5f, Movie13). Together, the observations of tissue remodeling of embryos developing in vivo and in vitro indicate that rosettes play a central role in pro-amniotic cavity formation.

**Polarized Podocalyxin exocytosis during rosette resolution and cavities expansion**

To gain insight into the molecular mechanisms of polarized rosette resolution and fusion of cavities, we generated a spatial transcriptome map of post-implantation embryos by generating sequential sections of embryos along their proximo-distal axis at successive developmental stages and performing RNA sequencing (Fig. 6a; Table S1). These analyses revealed three distinct cell populations corresponding to distal visceral endoderm, epiblast and extra-embryonic ectoderm. The identity of these tissues was confirmed by examining the spatial expression pattern of epiblast (Pou5f1) and extra-embryonic ectoderm (Cdx2) markers (Fig. S6a-b). Additionally, Wnt3 transcripts were detected in the distal extra-embryonic ectoderm and proximal epiblast and T/Brachyury transcripts were detected in the distal extra-embryonic ectoderm of E5.75 embryos (Fig. S6c) as described previously, highlighting the sensitivity of this method.

Analysis of differential gene expression between embryonic and extra-embryonic regions revealed the upregulation of 764 genes in the E5.25 embryo extra-embryonic compartment; 1119 genes in the E5.5 embryo extra-embryonic compartment; and 306 genes in the E5.75 embryo extra-embryonic compartment (Table S1, Fig.S7a). Importantly, functional enrichment analysis revealed that several genes involved in integrin-mediated signalling were specifically upregulated in the extra-embryonic compartment during stages II-III (Fig. S6d, Table S1). This is in accord with our results showing that ECM/integrin signalling drives extra-embryonic tissue polarization and rosette formation.

In accord with the requirement for Rab11-mediated exocytosis and the reorganization of exocytotic vesicles in lumen formation, we found several regulators of exocytosis and vesicle organization to be upregulated in the extra-embryonic compartment during pro-
amniotic cavity formation (Fig. 6b, Table S1). Specifically, expression of Rab11A, the main mediator of exocytosis during lumen formation, was enriched in the extra-embryonic compartment, suggesting that Rab11-mediated apical exocytosis is involved in this process.

In MDCK cells, lumenogenesis requires Par/aPKC mediated cell polarization and the concerted activity of Rab GTPases, to direct the polarized exocytosis of Podocalyxin. Examination of expression dynamics of genes involved in the regulation of Podocalyxin exocytosis revealed that they were upregulated in the extra-embryonic compartment either at one or both stages of tissue remodeling (II, E5.25 or III E5.5), during which time the transcriptional profile of the extra-embryonic lineage differs from stage IV-V (E5.75) (Fig. 6c; Fig. 7a; Fig. S7b). Together this suggests that Podocalyxin exocytosis plays a role during pro-amniotic cavity formation.

In agreement with the transcriptome analysis, we found Podocalyxin positive exocytotic vesicles in all cells of the extra-embryonic compartment at E5.5, whereas in the embryonic compartment Podocalyxin was present only at the apical side of cells (Fig. 7b). These Podocalyxin vesicles showed a polarized pattern of secretion, along the borders of polarized tracts connecting rosettes with cavities, in both hybrid and extra-embryonic rosettes (Fig. 7c-d). After fusion of the cavities, the extra-embryonic ectoderm cells not contributing to the cavity still contained Podocalyxin exocytotic vesicles (Fig. 7e). Polarized cell intercalation resulted in the formation of a pseudostratified epithelium in the extra-embryonic compartment in which all the extra-embryonic ectoderm cells faced the cavity (Fig. 8a, Fig. S8, Movie 14). At this point all the cells facing the cavity were negative for exocytotic vesicles (Fig. 8b). These results indicate that whereas polarized rosette resolution drives the initial fusion of cavities, further tissue remodeling driven by intercalation completes the process during the final step of pro-amniotic cavity formation.

Discussion

Upon implantation, the blastocyst transforms into the egg cylinder, a structure of an entirely different architecture. We show that this drastic remodeling is driven by a series of major morphogenetic events that we break down into five stages: stage I) polarization and lumenogenesis of the embryonic tissue and generation of the extra-embryonic tissue; stage II) the folding of the extra-embryonic tissue leading to its lumenogenesis and formation of a hybrid rosette on the embryonic/extra-embryonic boundary; stage III) remodeling of the
embryonic tissue and formation of multiple 3D rosettes within the extra-embryonic tissue mediated by ECM-β1-integrin signalling; stage IV) the polarized resolution of extra-embryonic rosettes mediating expansion of embryonic and extra-embryonic cavities mediated by Rab11/Podocalyxin vesicles exocytosis; stage V) the fusion of embryonic and extra-embryonic cavities and polarized cell intercalation resulting in the formation of a unified pro-amniotic cavity (Fig. 8c).

Two-dimensional rosettes have been described to contribute to tissue re-arrangements during morphogenesis in many model systems. Three-dimensional rosettes on the other hand, were shown to contribute to tissue shaping by generating self-contained central lumens rather than by rearranging cells. Our detailed analysis of embryos developing in vivo as well as time-lapse imaging of embryos developing in vitro indicate that whereas extra-embryonic ectoderm rosettes have the characteristics of 3D epithelial rosettes, they do not form self-contained lumens but, instead, form and resolve in a polarized manner to permit extension of cavities through the compact extra-embryonic tissue. The spatial and temporal profile of morphogenetic reorganization accords with progressive resolution of rosettes from the embryonic to the extra-embryonic lumen to form the pro-amniotic cavity.

Our results indicate that resolution of extra-embryonic ectoderm rosettes is preceded by re-orientation of cell polarity and expansion of the apical domain in a subset of cells. This creates polarized tracts along cell interfaces connecting lumens with the centers of rosettes. This re-orientation of cell polarity is followed by polarized exocytosis and lumen extension, as Rab11/Podocalyxin vesicles become targeted to the rosettes’ polarized tracts. Podocalyxin is a negatively charged sialomucin causing membrane repulsion and therefore its secretion at these sites could indeed result in rosette resolution. Since we observe that each rosette is exposed to the cavity before its resolution, it is possible that osmotic pressure from the cavity fluid contributes to membrane separation during rosette resolution. Thus, a model proposing that rosette resolution driven by exocytosis mediates expansion of the cavities is more in line with our data than mechanisms described for lumen expansion in other systems.

In summary, the detailed developmental, cellular and molecular characterization of the morphogenetic steps involved in the reorganization of the blastocyst into the egg cylinder structure leads us to propose a novel mechanism for cavity fusion that is mediated by tissue rearrangements governed by the formation and polarized resolution of multiple multicellular 3D rosettes. Our model suggests that polarized resolution of these cell arrangements mediates
the remodeling of the whole embryo leading to fusion of embryonic and extra-embryonic cavities. Reorientation of apico-basal polarity, cell rearrangement and the expansion and fusion of cavities have been described during development of different organisms\textsuperscript{27-30} and therefore it is likely that the morphogenetic processes leading to cavity formation we describe here might represent a common molecular and cellular mechanism utilized in processes other than mammalian embryogenesis.

**Author Contributions**

N.C and C.K designed and carried out the experiments and data analysis. A.W contributed to embryo live imaging. G.C and G.P performed the embryo cryosection, laser microdissection and library construction experiments for RNA-seq. R.W carried out the RNA sequencing analysis. N.J supervised the work related to spatial transcriptome analysis. M.Z.G conceived, supervised the study and wrote the manuscript with the help of N.C and C.K.

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**Methods**

**Embryo recovery and culture:** Mice were kept in the animal house in accordance with national and international guidelines. All experiments have been regulated by the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB). Experiments were approved by the Home Office (Licence number: 70/8864). Animals were inspected daily and those that showed health concerns were culled by cervical dislocation. Implantation stage embryos were recovered from wild type F1, MF1 or CD1 females mated with mTmG\textsuperscript{18}, LifeAct-GFP\textsuperscript{17} and wild type males. Pre-implantation embryos
were flushed out from the uteri as previously. Post-implantation embryos were dissected out of the deciduae and transferred into IVC2. After recovery, the embryos were transferred into drops of IVC2 (1 embryo/drop) covered with mineral oil and cultured according to the experimental designed. For the enzymatic removal of the BM, Collagenase IV (500ug/ml) was used for 3-5 hrs in IVC2 and then replaced with 50ug/ml for overnight culture. Embryos were cultured at 37°C in 5% CO2.

**Immunostaining:** Embryos were fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature. Post-implantation embryos were permeabilized for ~12-20 min in 0.3% Triton X-100 / 0.1M Glycin in PBS. The primary antibodies were then added to the blocking buffer (0.1% Tween-20 / 10% filtered FCS in PBS) and embryos incubated overnight at 4°C. Embryos were then incubated for 3-4 hrs at room temperature with secondary antibodies in blocking buffer. All washes were done in filtered PBS + 0.05% Tween-20 (PBST). Pre-implantation embryos were permeabilized in 0.5% Triton X-100 in PBS for 20 min at room temperature. Primary antibodies were added in the blocking buffer (3% BSA in PBS + 0.1% Tween-20) and embryos incubated overnight at 4°C. For visualizing exocytotic vesicles through immunofluorescence permeabilization was performed with 0.1% (w/v) Saponin (Sigma S7900) + 0.2% (w/v) gelatin (Sigma G7765) + 5mg/ml BSA for 30 min at room temperature. Primary and secondary antibodies were prepared in 0.2% (w/v) gelatin + 0.01% (w/v) Saponin. Primary antibodies were added to the sample overnight at 4°C while secondary antibodies were added for 2 hrs at room temperature after washing 3 times in PBS.

Primary antibodies used: ZO-1 (1:200; Thermofisher Scientific, 33-9100), E-cadherin (1:300; Thermofisher Scientific, 13-1900), aPKC (1:100; Santa Cruz Biotechnologies, sc-216), Oct4 (1:400; Santa Cruz Biotechnologies, sc-5279), GM130 (1:200; BD, 610822), Laminin (1:400; Sigma, L9393), Cdx2 (1:200; Biogenex, MU392A-UC), β1-integrin (1:50; Ha2/5; BD, 561796), active β1-integrin (1:50; 9EG7; BD, 553715), Ap2γ (1:300; Santa Cruz Biotechnologies, sc-8977), Eomes (1:400; Abcam, ab23345), Elf5 (1:400; Santa Cruz Biotechnologies, sc-9645), Podocalyxin (1:300; R&D systems, MAB1556), Pard6b (Santa Cruz Biotechnologies, sc-67393), pMLC (1:100; Cell Signalling Technologies, 3671P), Collagen IV (1:100; Millipore, AB769), HSPG2 (1:100; Millipore, MAB1948P), Rab11a (1:100; Cell Signalling Technologies, 2413S) Anxa2 (1:700; Abcam, ab41803). Secondary antibodies in pre-implantation blocking buffer were then added to the embryos for ~2 hrs at room temperature. Secondary antibodies used: Alexa 647 donkey anti-rabbit (1:500; Thermofisher Scientific, A31573), Alexa 594 donkey anti-rat (1:500; Thermofisher
Scientific, A21209), Alexa 568 donkey anti-mouse (1:500; Thermofisher Scientific, A10037), Alexa 488 donkey anti-mouse (1:500; Thermofisher Scientific, A21202), Alexa 488 donkey anti-goat (1:500; Thermofisher Scientific, A11055), Phalloidin 488 (1:500; Thermofisher Scientific, A12379). All washes were done in PBS + 0.1% Tween-20. Nuclear staining: minimum 15 min incubation in DAPI + PBS (5mg/ml).

**Imaging:** Freshly recovered and fixed embryos were imaged on a Leica SP5 or SP8 confocal microscope. For live imaging a multiphoton Leica SP8 was used. The wavelengths used for 2-photon excitation fluorescence (2PEF) when imaging mTmG (unconverted; Tomato) and LifeAct (GFP) were 1040nm and 910nm respectively.

**Laser ablation** was carried out on two-photon microscope (LaVision BioTec TriM Scope II). A multiphoton Insight DeepSee dual-line laser tuned to 920 nm was used to perform ablations. A region of interest was set to cell-cell interface and scanned at 3 different Z planes (1um) with the multiphoton laser set at 70% transmission with a pixel dwell time of 9.1μs. Images were taken immediately before, after and every 1 min following ablation.

**Image processing and analysis:** Fiji image processing software was used while 3D reconstructions of cells were carried out on 3D Slicer software using manually segmented images from Fiji.

**Cell culture:** Unconverted Confetti TSCs derived in defined conditions on fibronectin were used for TSCs aggregate experiments. TSCs were maintained and passaged under defined conditions on fibronectin-coated wells as described previously. The cells were passaged when they reached ~70% confluency (normally once every three days) and medium was changed the day after passaging and then every other day. The cells were kept at 37°C in 5% CO₂.

**Trophoblast stem cell aggregates:** TSCs were incubated for 2 min at 37°C in 0.05% trypsin-EDTA (Invitrogen) when they reached confluency. Trypsinization was stopped by adding TS medium (RPMI 1640 (Sigma), 20% FCS (GIBCO), penicillin/streptomycin (50μm/ml) (GIBCO), Sodium pyruvate (1Mm) (GIBCO), β-mercaptoethanol (100uM), L-glutamine (2mM) (GIBCO)). The cell suspension was collected and spun down (1000 rpm for 5 min). The supernatant was discarded, and the pellet re-suspended as clumps in defined culture medium prior to plating in Matrigel (BD, 356230). Using a haemocytometer the cell density was worked out and then appropriate volume of cell suspension was aspirated to suspend ~20000 cells/Matrigel drop. The cell suspension was spun down (1000 rpm for 5 min) and
supernatant removed. The TSCs in the pellet were gently re-suspended as clumps in a volume of Matrigel corresponding to 20ul / drop before plating each 20ul drop in one well of an ibiTreat microscopy plastic μ plate (Ibidi). After 2 min of incubation at 37°C to allow polymerization of the Matrigel, the media were added according to the designed experiment. For cells grown in the absence of Matrigel, cells were plated on a non-adherent suspension culture plate (CELLSTAR, 662 102). The cells were kept at 37°C in 5% CO₂.

**Embryo laser capture microdissection and RNA isolation:** The spatial transcriptome of embryos was obtained according to the Geo-seq methods. In brief, E5.25, E5.5, E5.75 MF1 embryos in deciduas were embedded in OCT compound and cryo-sectioned serially at 15 μm along the proximodistal embryo axis. Serial sections were mounted on polyethylene terephthalate-coated slides. Frozen sections were allowed to thaw at room temperature and then dehydrated in ice-cold 100% ethanol. Fixation was performed in 75% ethanol, then the slides were stained with 1% cresyl violet acetate solution (Sigma-Aldrich, prepared in 75% ethanol), dehydrated in a series of 75%, 95%, 100% ethanol (30 secs for each step), and finally subjected to LCM on an MMI Cellcut Plus system (MMI, Zurich, Switzerland).

Approximately 20 cells in each section were harvested by LCM. Cell samples were lysed in 50 μl of 4 M guanidine isothiocyanate solution (GuSCN; Invitrogen, 15577-018) at 42°C for 10 min. The volume of the lysate was adjusted to 200 μl by nuclease-free water, and was further concentrated by ethanol precipitation in the presence of 1/10 volume of acetate sodium (pH 5.7, 3 M; Ambion) and 2 μl of carrier glycogen (20 mg/ml; Roche). Total RNA pellets were dissolved in lysis solution and used as a template for low-cell number RNA-seq.

**RNA-Seq data pre-processing:** Raw reads were evaluated the quality with the FASTQC. Density distribution of gene expression for all samples were also plotted to assess whether there are inconsistent samples. Raw reads were mapped to mm10 version of mouse genome using Tophat2 v2.0.4 program. We calculated fragment per kilobase per million (FPKM) as expression level using Cufflinks v2.0.2 with default parameters. Genes with the FPKM > 1.0 in at least one sample across all samples were retained for further analysis. Finally, the expression levels were transformed to logarithmic space by using the \( \log_2(FPKM+1) \).

**Hierarchical clustering** and **PCA** are based on all the expressed genes as described in the RNA-Seq data pre-processing.
Differentially expressed genes (DEGs) analysis between epiblast (EPI) and extraembryonic ectoderm (ExE) were identified using RankProd\(^{36}\) with P value < 0.05 and fold change > 1.5.

**Functional enrichment analysis** of gene sets with different expression patterns was performed using the Database for Annotation, Visualization and Integrated Discovery v6.8 (DAVID v6.8\(^{37}\)).

**Gene expression visualization**: to visualize the genes expression patterns in each embryo section, we wrote a program for visualization in MATLAB (version: 2015a). To analyze the genes expression dynamics during development (E5.25, E5.5, E5.75), we normalized the three sequencing batches to the same standard using ComBat\(^{38}\), then ran the MATLAB program.

**Statistics and Reproducibility**

Statistical analyses were performed using GraphPad Prism 6.0 software. Embryos were randomly allocated to control and experimental groups. Sample size was determined based on previous experimental experience. Investigators were not blinded to group allocation. Quantitative data are presented as mean ± s.e.m. Quantitative data were analysed using a two-sided unpaired Student’s \(t\)-test and qualitative data were analysed with \(\chi^2\) test. Unless otherwise noted, each experiment was performed at least three times.

**Data availability:**

RNA–seq data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession code GSE110808. Source data for Fig. 1b, 2c,2e,2g,2i,2j,2l,3c,3h and Supplementary Fig. 3c,3d have been provided as Supplementary Table 2. Source data for Fig.1g,h; Fig 3e,f; Fig. 4d,f; Fig. 5c,d and Fig. S3c are provided as supplementary movies. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

**References**


Figure legends

Figure 1: Stages of embryo remodeling and pro-amniotic cavity formation

(a) Morphological staging of early post-implantation embryos based on the development of a pro-amniotic cavity; Arrowhead points to apical side of extra-embryonic ectoderm (ExE) cells at the boundary and arrows point to the ExE cells facing the epiblast (EPI) cavity; circled portion of stage III embryo highlights the invading EPI cavity into the ExE. n=142 embryos. (b) Quantification of the distance between the EPI and ExE cavities during pro-amniotic cavity formation. Two sided unpaired student’s t-test; ****P<0.0001; mean±SEM; n= 21 stage II, 28 stage III and 20 stage IV embryos. (c) Percentages of embryos found in specific pro-amniotic cavity stages according to embryonic day staging system; =73 E5.5 + 31 E5.75 embryos.(d) Schematic representation of the five major stages of pro-amniotic cavity formation as identified through analysis of freshly recovered embryos. VE, visceral endoderm; ECM, extracellular matrix; EPC, ectoplacental cone. (e) Magnification of the area at the EPI-ExE boundary from d showing the reorientation of EPI boundary cells (red arrows). (f) Extra-embryonic cavity formation. Each stage of extra-embryonic cavity (cyan
asterisks) formation was characterized by localization of tight junction protein ZO-1 (white arrowheads) and enrichment of E-cadherin (yellow arrowheads) as well as cell shape changes accompanied with bending of the tissue (white arrows). Yellow asterisk indicates embryonic cavity. White dots mark ExE proximal cells acquiring columnar morphology. Red dots: cell apical site; P: proximal; D: distal. n=25 embryos. (g) Stills from a time-lapse movie showing apical constriction driven tissue bending during extra-embryonic cavity formation. Red arrowheads mark apical actin enrichment during apical cell constriction which leads to cell ingression and tissue bending. Dotted line: cavity. Segmented panel: Red outline marks cells undergoing apical constriction. Yellow line: tissue outline. Note that these cells ingress inducing tissue bending and cavity formation. Green outline: lateral cells. n=5 embryos. (h) Stills from single cells in (g); Cells show apical surface area reduction before ingression. Enrichment of apical actin (red arrowheads) coincides with apical constriction. Scale bars=20um.

Figure 2: Extra-cellular matrix/β1-integrin signalling is essential for extra-embryonic ectoderm polarization and morphogenesis

(a) Polarity pattern assessment in stage II E5.5 extra-embryonic ectoderm (ExE) based on Golgi position (GM130); Schematic: position of Golgi in manually segmented and position-based colour coded ExE. White dots: Golgi. n=10 embryos. (b) Cell morphology of outside (i) and inside (i’) cells as shown by 3D cell segmentation. (c) Cell aspect ratio of outside vs inside ExE cells. Two sided unpaired student’s t-test; ****P<0.0001; mean±SEM; n=50 cells, 6 E5.5 embryos. (d) TSCs grown as aggregates in Matrigel for 48 hrs in the presence of N2B27 and FGF2, stained for tight junction marker ZO-1 or golgi marker GM130, F-actin and the TSC marker Eomes. n=3 biological replicates. (e) Cell aspect ratio of outside (n=31) vs inside cells(n=36) in TSCs .Two sided unpaired student’s t-test; ****P<0.0001; mean±SEM. (f) Cell morphology of outside ExE cells in control and COLIV treated embryos. Embryos in each group are shown at two different z slices and a zoomed image of outside cells is displayed on right of each image. n=3 biological replicates. (g) Comparison of cell aspect ratio of outside cells in control and COLIV-treated embryos. Long axis = axis perpendicular to basement membrane; short axis = axis perpendicular to long axis at half length; Two sided unpaired student’s t-test; ****P<0.0001; mean±SEM; n=60 cells, 6 Control and 10 COLIV treated embryos. (h) TSC aggregates cultured in Matrigel in the presence of N2B27 and FGF2 for 48 hrs. β1-integrin function blocking antibody (Ha2/5) was added at 24hrs. n=3 biological replicates. (i) Quantification of apical ZO-1 subcellular
localization (apical ZO-1 intensity/basolateral ZO-1 intensity) in control (n=40 cells) and β1-integrin function-blocking antibody-treated TSC aggregates (n=40 cells); Two sided unpaired student’s t-test; ****P<0.0001; mean±SEM. (j) Comparison of cell aspect ratio of outside cells in control (n=51 cells) and β1-integrin function-blocking antibody-treated TSC aggregates (n=32 cells) Two sided unpaired student’s t-test; ****P<0.0001; mean±SEM. (k) Basement membrane disruption after treatment with COLIV leads to failure of pro-amniotic cavity formation. (l) Quantification of pro-amniotic cavity formation efficiency in control and COLIV-treated embryos; χ² test ; ****P<0.0001. For (k) and (l) n= 27 control and 24 COLIV-treated embryos. n=3 biological replicates. Scale bars=20um.

Figure 3: Extracellular matrix-dependent 3D rosette formation throughout the extra-embryonic ectoderm

(a) Representative examples of E5.5 embryos showing formation of extra-embryonic ectoderm (ExE) rosettes as identified by cell morphology. n=20 embryos. (b) E5.5 embryos stained to reveal tight junctions (ZO-1), polarity (aPKC, Par6) and actomyosin markers (F-actin, pMLC). Magnified images centered on ExE rosettes (outlined by dashed line). Arrows: rosette center. n=20 embryos. (c) Average number of ExE rosettes observed per stage of pro-amniotic cavity development (17 stage II, 21 stage III, 23 stage IV, 10 stage V embryos; Two sided unpaired student’s t-test; ***P=0.0002; mean±SEM. (d) Representative image of an E5.5, stage III embryo. Orthogonal projections are displayed on the right and bottom of the image. Asterisks label bipolar cells in the rosette either having a second apical side in the ExE cavity or the epiblast cavity. Rosette schematics are displayed on the bottom right with red dots indicating bipolar cells. Inset shows magnified image of the rosette. n=10 embryos. (e) Rosettes identified and segmented at different z optical sections from a single E5.5, stage III embryo. Segmented panel: Segmented cells for each rosette are differentially and arbitrarily colour-coded for 3D rotation clarity. 3D Rendering panel: Each cell is also coded with a roman numeral (rosette 1: i – vi; rosette 2: i’ – vi’). Rotation and magnification of rosette centres indicated by the dashed circle demonstrate absence of self-contained lumen as apical surface of cells meet at rosette centre. (f) Combined 3D rendered rosettes in the same spatial frame to assess cell sharing. Shared cells are highlighted. Both colour and number coding is retained from (e) and displayed in this panel. Magnification of rosette centres indicated on top right of each box. (g) ExE rosette formation in control and COLIV treated embryos. Orthogonal slices are projected on the right and bottom of the images. n=12 embryos;3 biological replicates. (h) Percentage of embryos with or without identified ExE
Rosettes in control and COL IV-treated groups. $\chi^2$ test; ****P<0.000; n= 12 control and 12 COL IV treated embryos; 3 biological replicates. (i) Model of ECM/β1-integrin-mediated signalling mechanism for polarity establishment and rosette formation in ExE. Scale bars=20um.

Figure 4: Epiblast-extra-embryonic ectoderm hybrid rosette polarized resolution drives epiblast reorganization

(a) Two representative examples of hybrid rosette forming at the EPI (filled dots)/extra-embryonic ectoderm (ExE) (hollow dots) boundary. Arrowhead: pMLC enrichment. Insets show EPI/ExE boundary. Dashed lines in magnified images indicate ExE cells where solid lines indicate EPI cells. Dashed outline in example II points to an ExE rosette. YZ orthoslice is projected on the right of example II. n=10 embryos. (b) Peri- and early post-implantation embryos stained for laminin to assess integrity of basement membrane between EPI and polar trophectoderm (pTE)/ExE (yellow arrowheads). White dots indicate pTE cells. n=15 embryos. (c) Freshly recovered embryos at different stages of hybrid rosette development. Rosette resolution is displayed in a temporal sequence from left to right. solid outlines: EPI cells; dashed outlines: ExE cells; grey outline: EPI cavity. Arrow: pMLC puncta belonging to the resolved rosette. Inset in right panel shows a different Z slice where the re-oriented EPI is evident. n= 8 embryos. (d) Stills from a time-lapse movie of LifeAct-GFP E5.5 (late Stage II) embryo showing EPI/ExE hybrid rosette resolution and EPI reorganisation. Green box magnifications indicate the centre of hybrid rosette, made up of EPI (blue dots) and ExE cells (red dots) as it loses the actin enrichment (yellow arrowheads) in preparation for resolution. Green box at 80min time-point highlights an ExE rosette in proximity to EPI cavity. Red dots indicate rosette cells. Right panel: Manually segmented version of the time-lapse movie focusing on the resolving hybrid rosette and a forming EPI cavity proximal rosette (green in second row).n= 3 embryos. (e) Representative E5.5 embryo with EPI/ExE hybrid rosette formed at the EPI/ExE boundary. Arrowhead: rosette centre; Arrows: Polarized tract; Hollow dots: ExE cells; Filled dots: EPI cells. n=6 embryos. (f) Left panel: Still images from time lapse movie after laser ablation of adhesion sites connecting the EPI cavity with hybrid rosette centre. Red line: ablation site. Right panel: Magnified images of the boundary region before and after ablation. Purple region with green outline shows the EPI cavity.
Figure 5: Rosette formation/resolution-mediated pro-amniotic cavity formation

(a) Representative example of stage III E5.5 embryo. Magnified images show extra-embryonic ectoderm (ExE) rosette in proximity to epiblast (EPI) cavity with segmented counterparts. Arrows point to polarized tracts (assessed with tight junction marker ZO-1) extending from EPI cavity to centre of rosette before EPI cavity progression. n=10 embryos.

(b) Representative examples (I & II) of stage IV E5.5 embryos. Magnified images show resolved ExE rosette in proximity to EPI cavity with segmented counterparts. Arrows point to expanded apical domain (assessed with polarity marker aPKC) of ExE cells after rosette resolution and EPI cavity progression. Asterisks indicate cells which lost their connection upon rosette resolution. n=10 embryos.

(c) Left panel: Stills from a time-lapse movie of mTmG stage III embryo transitioning to stage IV of pro-amniotic cavity development. Box (i) focuses on the EPI-ExE boundary and segmentation analysis is shown in middle panel. Cells are manually segmented and differentially and arbitrarily colour-coded for clarity. Extraction of segmented-only data is displayed next in right panel for the period of 40–100min where formation and resolution of rosette is observed. Arrows in right panel indicate rosette resolution and EPI cavity progression. n=3 embryos.

(d) Stills from a time-lapse movie of Lifeact-GFP embryo showing ExE cavity extension through polarized rosette resolution. Note the loss of actin enrichment from polarized tract (arrows) connecting the cavity with the rosette centre (arrowhead) before rosette resolution. Yellow filled area; ExE cavity; Magenta dots: rosette cells; Green dots; cells that lost adhesion; n=3 embryos. Scale bars=20um.

Figure 6: Spatial transcriptome analysis of pro-amniotic cavity formation

(a) Experimental strategy: cells were captured by laser capture microdissection along the proximodistal embryo axis and analysed by RNA-seq (Methods). Purple: epiblast (EPI); Cyan: extra-embryonic ectoderm (ExE); Yellow: VE. P: proximal; D: distal. (b) Differential gene expression analysis heat maps for gene ontology terms: exocytosis, vesicle organization. E5.25: sections 2-4 EPI, sections 6-8 ExE; E5.5: sections 2,4,5 EPI, sections 6-11 ExE; E5.75 sections 3-8 EPI, sections 10-19 ExE. Purple outlines highlight genes previously reported to be involved in lumen formation. (c) Expression pattern of selected genes during post-implantation development. Left: Schematic showing the position and tissue identity for each...
sequenced section (numbers on the left of the plot) in E5.25, E5.5 and E5.75 embryos. Gene expression is presented as colour coded corn plot. Colour coding represents log₂ (FPKM+1) values. FPKM: fragment per kilobase per million.

Figure 7: Polarized pattern of Podocalyxin exocytosis during rosette resolution and cavities expansion

(a) Annexin A2 localization in a representative E5.5 embryo. n=10 embryos. (b) Rab11/Podocalyxin localization in E5.5 embryo. Arrows show Rab11/Podocalyxin positive exocytotic vesicles. n=10 embryos. (c) Representative E5.5 embryo with epiblast (EPI)/extra-embryonic ectoderm (ExE) hybrid rosette (dots). Lower panel shows magnified images of EPI/ExE boundary. Arrow indicated the polarized tract connecting the EPI cavity with the rosette centre. Arrowheads show Rab11 and Podocalyxin positive vesicles docking cell/cell interfaces forming the polarized tract. n=5 embryos. (d) Representative Z-stacks of an E5.5 embryo. Upper panel shows an ExE rosette being connected with the EPI cavity via polarized tract (arrows). Rab11/Podocalyxin positive vesicles are docked (hollow arrowhead) or polarized along the polarized tract (filled arrowhead). Lower panel shows ExE rosette being connected with the ExE cavity via polarized tract (arrows). Rab11/Podocalyxin positive vesicles are docked along the polarized tract (hollow arrowhead). n=5 embryos. (e) Maximum intensity profile image of a representative E5.75 embryo just after cavities fusion. A unified cavity (arrows) spans the embryonic and extra-embryonic compartment. Exocytotic vesicles are present in extra-embryonic cells not directly in contact with the cavity (arrowheads). n=5 embryos. Scale bars=20um.

Figure 8: Cell intercalation during the final step of pro-amniotic cavity formation

(a) Time-lapse movie extraction of an mTmG E5.5 (stage II) embryo with segmented cavities (purple) showing a cell (green) intercalating towards the basement membrane. n=2 embryos. (b) Representative example of an E6.25 embryo. At this stage all ExE cells contribute to the unified pro-amniotic cavity and ExE is a pseudostratified epithelium. All ExE cells are negative for intracellular exocytotic vesicles. n=10 embryos. (c) Model of pro-amniotic cavity formation. During stage I, the epiblast cavity forms through hollowing and the polar trophoderm proliferates to form the extra-embryonic ectoderm (ExE). The ExE cells
undergo apical constriction, which induces the formation of the ExE cavity through tissue folding. At stage II, in the presence of embryonic and ExE cavities, a hybrid rosette forms at the embryonic/extra-embryonic boundary. Polarized resolution of this rosette results in remodeling of embryonic compartment and progression to stage III. After, extra-embryonic rosettes form and undergo polarized resolution, the embryonic and extra-embryonic cavities extend. At stage IV the extended cavities merge. At stage V, a newly-formed unified pro-amniotic cavity spans the egg cylinder. Scale bars=20um.