A uterus-inspired 3D niche drives embryo development beyond implantation

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Article

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Abstract

The mammalian embryo must undergo dramatic morphogenetic changes to invade the uterine endometrium and achieve implantation. Thus, recapitulation of implantation using in vitro systems is crucial for revealing the mechanisms controlling early development and the main problems compromising human fertility. Experimental systems based on two-dimensional (2D) platforms cannot fully recapitulate the in vivo 3D microenvironments of the embryo. Therefore, here we use collagen grafted onto polydimethylsiloxane (PDMS) based on the uterine mechanics and microstructure to establish a uterus-inspired 3D niche (U3N). Our U3N enables mouse embryos to form egg cylinders at high rate and reach the developmental stages of heartbeat. Moreover, a unique interface forms between the embryo and collagen, showing the invasion of trophoblasts into collagen fires, which simulate the developmental process of implantation. Our findings highlight embryo-substrate interaction as a key characteristic of post-implantation development in vitro and as an important design parameter of 3D conditions for embryo culture.

Introduction

Implantation of the embryo into the uterus represents one of the key events of mammalian development[1]. Trophectoderm (TE) cells of the blastocyst make the first contact with the uterine epithelium for implantation. The contact part of the embryo is defined as mural trophoblast and the opposite part far from the uterus is called as polar trophoblast. When mural trophoblast attached to uterus, the epithelial cells of which become receptive for embryos with the stimulation of progesterone and estrogen. Subsequently, the mural trophoblast cells develop into trophoblast giant cells which secrete matrix metalloproteinases to rebuild the extracellular matrix (ECM) for the invasion of embryos into uteri on 4.5 days post coitum (E4.5). The TE of embryos differentiate into extraembryonic ectoderm (ExE) and epiblast cells (EPI) of the embryo develop into pro-amniotic cavity after implantation. The mature amniotic cavity is formed from EPI and ExE in egg cylinder on the stage of E5.5-E6.5 followed with a series of developmental events including gastrulation, first appearing of allantoic, amniotic fold, first brachial arch etc. The incorrection of any of these events will lead to abnormality of the embryo development and even pregnancy failure[2]. Yet, the development of post-implantation embryos remains poorly understood because the embryo becomes closely tied to the mother and is embraced by the maternal uterine tissue of the decidua, which poses a challenge to the direct observation of embryonic organogenesis *in utero*[3,4]. Culturing whole-embryo cultures on top of a matrix which mimic the uterus serves as an excellent model to study embryo morphogenesis and organogenesis [5-10].

Early studies have cultured mouse embryos on top of a fibre network of bovine eye[11] from blastocyst stage to 5.0 days post coitum (E5.0). Since then, a series of *in vitro* culture (IVC) systems have tried to simulate the conditions of womb via different approaches. These include tissue culture plates with various coatings such as collagen, laminin or fibronectin[12,13]; co-culture with primary cells isolated from the uterus[14]; co-culture with somatic cells, such as fibroblasts and Vero cells[15]; small culture dishes

coated with polyacrylamide hydrogel on the bottom of the glass plate[16]; and matrigel basement membrane matrix[17,18]. These studies have found that specialised materials such as collagen allowed better embryo development[19]. However, rare studies focus on the development beyond implantation and the accurate simulation of uterine environment remains a massive challenge because there are great changes in terms of cell migration and tissue morphology for the embryo and the uterus together, in which there are also complicated physical and chemical factors involved.

Biological materials offer the possibility to manipulate mechanical hardness, elasticity and chemical modifications to affect key cell behaviours, such as cell migration, proliferation and differentiation[20-24]. The mechanical properties of matrix could also influence embryo development. The modulus for the oviduct and uterine epithelium is approximately 100-1000 Pa, with a difference of almost six orders of magnitude from the conventional polystyrene culture plate, which is about 1 GPa[25,26]. There is an urgent need to better understand the relevant mechanisms and design optimal materials inspired by uterine environments to accomplish better embryo culture *in vitro*.

In this study, we established a uterus-inspired 3D niche (U3N) with a layer of collagen on a PDMS substrate, which closely mimic the physical modulus and porous structures of the uterus matrix. Adjacent radial microvilli of the embryo rearrange the collagen fibres at the interface and the implantation-related genes were also detected, suggesting this co-culture system is similar to the implantation *in vivo*. to create The U3N system drives embryo development beyond implantation to E8.5 *in vitro* (Fig. 1a, b) and the success of this approach was evidenced by the formation of key structures, including the head, the blood island of the yolk sac and the heartbeat. Furthermore, there was a higher ratio of the formation of egg cylinder and heartbeat for the embryos in U3N system.

Results

Construction of uterus-inspired 3D niche for better early embryonic development

In order to reproduce the mechanical and structural environment of the endometrium, we first measured the modulus of the endometrium and the uterine horn in adult mice (Supplementary Fig. 1a) and found the modulus ranges of the endometrium and the uterine myometrium are 2.37 ± 0.81 kPa and 0.41 ± 0.16 MPa, respectively. We then measured the modulus of 7.0 mg/ml collagen and PDMS with mixing ratio 10:1 (base to crosslinker) and they were 0.83 ± 0.13 kPa and 0.92 ± 0.10 MPa respectively and the commonly used Petri dish is 1 GPa as we know. Modulus of endometrium and collage were both at kPa levels, while myometrium and PDMS were both at MPa levels, suggesting a combination of collagen and PDMS can mimic the mouse uterus well (Fig. 1b).

The embryo-uterus synchrony is critical for successful implantation and subsequent development[27]. To better mimic the uterine, we carefully studied the macro and micro structure of the uterus matrix and found there were special patterns. The diameter of the fibre (D) and the size of the pore cross-sections (S) of the uterus matrix were 0.09 ± 0.02 μm and 0.72 ± 0.21 μm^2 (Fig. 1d). Then, we made porous hydrogel

from collagen with specific concentration (7.5 mg/mL) collagen with D = 0.12 ± 0.02 μm and S = 0.69 ± 0.17 μm^2, which was similar to the uterus matrix (Fig. 1d). Based on the above two points, we designed a U3N by grafting a layer of collagen (CO; 7.5 mg/ml; 46 ± 10 μm in thickness) onto the PDMS (PD; 496 ± 156 μm in thickness) and form the PDCO to the uterus during pregnancy (Fig. 1b and Supplementary Fig. 1).

We harvest E3.5 embryos from pregnant female mice and transferred them onto petri dish, PD or PDCO (Fig. 2a and Supplementary Fig. 2). This co-cutlre system was maintained in vitro for over 10 days (Supplementary Fig. 2a). Compared with embryos cultured on PD (16 ± 8%, n= 64 embryos), those grown on PDCO (36 ± 6%, n= 71 embryos) exhibited a higher ratio of two cavities (pre-promamniotic cavity and TE cavity) on day 3 of co-culture (Fig. 2b and Supplementary Fig. 2b). Additionally, there was a higher rate of formation of the egg cylinders (47 ± 15% for PDCO and 32 ± 11% for PD) and an enhanced developmental efficiency of the embryos with heartbeat (11 ± 5% for PDCO and 0% for PD) in the embryos on PDCO (Fig. 2b) compared with those on PD or CO alone (Supplementary Fig. 2). Furthermore, the embryos cultivated to E4.5 on PDCO were transferred into the uteri of pseudo-pregnant mice and then the uteri were collected on 6 days after transplantation. We could observe successful decidua formation (36.7%, n = 24 embryos), which was nevertheless not noticed in the PD group (Fig. 2c) (n = 29 embryos). The result confirms the necessity of establishing a uterus-like environment from both the macro (modulus of the uterus horn and endometrium) and micro (microstructure of the endometrium) perspectives.

Cell lineage segregation is crucial for early embryo development and is a criterion to define the quality of an embryo. For the reason, we performed single-cell RNA sequencing on embryos cultured on PD or PDCO and then analysis confirmed that the cell population of embryos grown on PDCO on day 2 of IVC is closer to E4.5 embryos *in vivo.* Although embryos grown on PD and PDCO had cell populations of trophoblast (TE), primitive endoderm (PrE), epiblast (EPI) and inner cell mass (ICM) (Fig. 2d-f and Supplementary Fig. 3), which was consistent with previous findings[28]. EPI gives rise to almost all of the fetal tissues and the statistics showed embryos in the PDCO group had more EPI and fewer TE cells than those in the PD group (Fig. 2f).

To further investigate the relationship between embryo development and the 3D substrate, we optimised U3N by adjusting the key parameters of collagen in terms of the thickness and concentration, based on which we designed a series of experimental groups and the schematic diagram and the abbreviation of the groups were shown in Supplementary Fig. 4. We first tested the effects of the thickness of collagen on embryo development. Our results suggested that higher rates of embryo with two cavities lead to a trend towards an increased rate of egg cylinder embryos on day 4 of IVC, although this was not statistically significant for the PDCO-t group, or a modified PDCO with the middle thickness of the top layer of collagen (Supplementary Fig. 5a, b). We then fixed the thicknesses of both collagen (46 ± 10μm) and PDMS (496 ± 156μm), but modified the concentration of collagen (5.0 mg/ml, 7.5 mg/ml, 10.0 mg/ml) and generated PDCO-L (5.0 mg/ml), PDCO-M (7.5 mg/ml) and and PDCO-H (10.0 mg/ml) (Supplementary Fig. 4). We observed that the formation rate of egg cylinder for PDCO-M, with a moderate

level of collagen concentration (7.5 mg/mL), was higher than those of other groups with either higher or lower concentrations at the same thickness of collagen (Supplementary Fig. 5c, d).

The above results all showed that the concentrations and the thicknesses of the collagen in the U3N system somehow determine the growth of the embryo. And the collagen at a concentration of 7.5 mg/mL and a thickness of 45.6 μm can best support embryo growth. In order to clarify why this is the truth, we collected the PDCO-t and PDCO-T with different collagen thicknesses and carefully examined gel porosity in terms of the surface and the section using scanning electron microscopy (SEM), adopting freeze-fracturing for internal analysis. The collagen precisely represented the uterus matrix in terms of the fibre diameter and pore area in the front and cross-sections at a concentration of 7.5 mg/mL and with a thickness of 45.6 μm (Supplementary Fig. 6). From this, we concluded that the modified PDCO with a moderate thickness of collagen (PDCO-t) allowed better embryonic development.

Interactions between peri-implantation mouse embryos and uterus-inspired 3D reconstructed environments

Epithelium–trophectoderm adhesion and interaction is the initiated stage of implantation. However, there were few reports describing the maternal-fetal interface using the ex-vivo long-term culture system [29-32]. We investigated whether there was invasion of embryos into the U3N system through confocal imaging of the embryos and the substrates. The embryos exhibited a flat morphology with a prominent nucleus on the surface of the PD after being attached, a significantly lower proportion of CDX2-positive cells aggregated (Fig. 3a). By contrast, the attached embryos on PDCO showed a dome-shaped morphology, with uniform nuclei and rearranged collagen fibres around the contact interface.

At the time of implantation, mural trophectoderm cells attach to the uterine luminal epithelium. TE-derived cells secrete a variety of proteinases to digest matrix materials and invade the uterus by remodelling the extracellular matrix (ECM) *in vivo*[33]. After blastocysts attaching to PDCO, TE-derived cells remodelled and invaded into the collagen gel (Fig. 3a and Supplementary Figs. 7), as observed in 3D imaging (Supplementary Fig. 7 and Supplementary Movie 1). Some part of embryo was under the surface of collagen, which was not observed for the embryo on PD (Supplementary Fig. 8 and Supplementary Movie 2). The embryo itself has distinct steroid or polypeptide secretions, leading to the maintenance of the pregnancy[34]. Therefore, we performed gene expression analysis which determined high expression of 9 types of hormone such as *Apln*, *Sct*, *Tspan33* and *Actn1* (Supplementary Fig. 9a). The expression of 12 implantation-related genes was also detected, such as *Emp2*, *Itgb4*, *Cdh1*and *Igf2* (Supplementary Fig. 9b).

To assess the real connection between embryos and materials during attachment and invasion, SEM imaging was performed and displayed the formation of radial microvilli where the embryos attached. A peculiar dynamic traction of collagen fibres was also found (Fig. 3b). By contrast, embryos did not grow out visible microvilli with slippy morphology on PD (n = 5) (Fig. 3b). We use TS cells to mimic TE cells, it was attempted to use the PDCO culture system to support mouse embryo stem cells (ESCs) and TE stem

cells (TSCs). The result showed that, relative to ESCs, TSCs and their derivatives showed an obviously similar microvilli structure to what we observed for the embryos on PDCO (Fig. 3c), which demonstrates that TE plays a prominent role in surface interactions when embryos contact collagen fibres. Furthermore, trophectoderms detailed characterized on day 2 of IVC to see the effects of 3D condition on embryo development in terms of staining of differentiation marker, mechanic properties and RNA expression analysis because trophectoderm is the distinct part of the embryo to be attached to the substrate.

We found that the appearance of embryos was different on the day 2 of IVC, the embryos on PD had a relatively larger flag spreading out than the ones on PDCO (Fig. 4a, b). Immunofluorescent imaging confirmed that the spreading areas induced by the interaction between the embryos and substrates were dominated by trophectoderm differentiation (TFAP2C positive, Fig. 4c)[35,36]. Cells with lower modulus were demonstrated with higher motility[37-39]. Atomic force microscopic (AFM) measurement discovered TE-derive cells with a higher modulus on PD than the ones on PDCO (Fig. 4d–f), which represented one of the reasons why cells could invade collagen. The differentially expressed genes of embryos on PD and PDCO showed that upregulated genes were enriched in negative regulation of the intrinsic apoptotic signalling pathway and the oxidation-reduction process (Fig. 4g). Another gene expression comparison indicated that the upregulated genes in embryos cultured on PDCO were mainly clustered on embryo implantation, such as *Fkpb4*, which is located in the decidualised stromal cells around the implantation site[40]; embryo organogenesis, such as *Wnt7b*, which is involved in placental development and angiogenesis[41]; *Grhl2*, which is related to forebrain development and cell proliferation[42,43]; *Coro1b*, which is correlated with *F-actin* and regulates cell migration[44,45]; and *S1pr1*, which improves the movement of smooth muscle cells, the progesterone receptor signalling pathway, cellular response to retinoic acid, negative regulation of intrinsic apoptotic signalling pathway and oxidation-reduction process, among other things (Fig. 4g). These results showed that the embryos on PD and PDCO were different in morphology, cell modulus, and gene expression, which initiated on the day 2 of IVC. Taken together, these data substantiated the influence of subtracted on the trophectoderms, which may further dominate the embryonic development.

Development of embryos supported by PDCO beyond gastrulation

In order to clarify the stage of development for embryos cultured on PDCO, we first evaluate the embryo morphology at distinct points during culture using standard microscopy according to comparison with the normal embryos *in vivo*. The embryos on PDCO could be cultured to the stage of E8.5 with typical structure at each point (Fig 5a). The patterns of morphogenetic cell lineage formation at each chronological had been also demonstrated in the cartoon figures (Fig 5b). Morphologically, red haematopoietic cells were visible in the yolk sac of embryos on day 8–10 of IVC (Supplementary Fig. 10a), and these may have been nucleated red blood cells. At the same time, an embryonic heartbeat was distinctively visible behind the pharyngeal of the body (Supplementary Fig. 10b and Supplementary Movie 3).

Furthermore, we stained the embryos with OCT4 for EPI, FOXA2 for PrE and CDX2 for TE on day 4 of IVC. The results of confocal imaging indicated that the embryos supported by PDCO exhibited the structure of typical egg cylinder on day 4 of IVC (Fig. 5a). Normally, embryos undergo a delay when cultured *in vitro* compared to those *in vivo*[46,47]. The embryos still experienced a delay in PDCO system, whereas which allowed embryo developing into the pre-gastrulation stage 1 day earlier compared with the other culture systems (Figs. 6a). When IVC reached the fifth day, the embryos manifested the asymmetric structures of amnion cavities, which implied that embryos had developed into early gastrulation stage and were similar to the E6.5 embryos *in vivo* (Figs. 6b). To further confirm the effects of long-term culture for embryos, the U3N system was also superior not only with regard to the morphology of embryos (Fig. 6b) but also for transcriptomic expression. Unsupervised clustering analysis of RNA-Seq data showed that comparable transcriptome characteristics were observed among the embryos on day 8-10 of IVC and the embryos of E7.5 and E8.5 (Fig. 6c). To assess cell fates over a period of development, we tested specific lineage markers of gastrulation and different organogenesis, including neurulation, yolk sac haematopoiesis and cardiogenesis. The embryos on day 8–10 of IVC also expressed more haematopoiesis-related genes, such as *Wdr5*, *Farsa*, *Rap1a*, *Myo1c*, *Bud31*, *Mrpl38*, *Calm2* and *Hspa4*, than E8.5 embryos (Fig. 6d). Transcriptome analysis also confirmed that the IVC embryos expressed cardiogenesis-related genes, such as *Acta2*, *Efhd2*, *Tpm1*, *Adk*, *Vdac2*, *Selenow*, *Pkp2* and *Fermt2* (Fig. 6e). Markers of nerve, primitive streak and notochord-related genes were also detected in the embryos on PDCO (Supplementary Figs. 10c, d and 11).

This work presents the application of 3D conditions in mediating embryo growth *in vitro* to the stage of organogenesis. The uterus-inspired 3D niche, U3N was created through combination PDMS and collagen and the optimization of the thickness and concentration collagen. We found that TE volume expansion was restricted over the attached substrate (XY pane) and exceeded into the collagen gel vertically, subsequently leading to high efficiency of embryo development. In contrast, there was larger extension area of TE when embryos cultured on PDMS with collagen coating (PDCO-C) but there was no vertical invasion, which resulted in lower efficiency of embryonic development. The invasion of the embryo through fibres, which authentically mimics early mouse embryonic development *in vivo*. It is known that TE-derived cells secrete matrix metalloproteinases and cathepsins to digest ECM and maternal cells, and embryos invade the uterus by remodelling ECM *in vivo*[33]. *In vitro*, we found that during embryo implantation, TE-derived cells were in close contact with collagen, and the collagen fibres were remodelled in a way that maybe similar to embryo implantation *in vivo*.

Simultaneously, the modulus differences among TE-derived cells on PD and PDCO (Fig. 4f) indicated a synergy between cells and cell substrates for stiffness[48]. It was observed that the pores in PDCO were larger than those in PDMS, facilitating the penetration of embryos embedded on PDCO (Fig. 3a). Therefore, micro/nano structure influences the movement of cells in a direction perpendicular to the surface or within the 3D structure; this result was also supported by single-cell RNA-seq profiling revealed Hippo-pathway genes with higher expression (Supplementary Fig. 10e), which also influence the

expression of CDX2[49], a critical gene for early embryo development and the differentiation of TE-derived cells[50,51].

Our findings also provides a solution to a conundrum of culturing embryos *in vitro* , which can determine the influence of the TGC formation on the later development of embryos [18,52]. Drastic changes in terms of embryonic structure and cell variation make it challenging to prolong normal development and to portray the precise status of the post-implantation embryo *in vitro*. Our 3D uterus-like structure enables new forms of research on the development of the yolk sac, and early embryonic organs, which can broaden knowledge of embryogenesis during post-gastrulation. In summary, the U3N exhibits an exquisite ability to promote embryo development *in vitro* in a way that produces a great rate of embryos reaching egg cylinder and heartbeat. Acceding to the opportunities for further investigation, we propose that the present platform could be useful tool for early developmental biology with novel mechanisms of cell fate determination, advanced transplantation therapy and tissue fabrication engineering.

Online Content

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Methods

Animal and ethical approval. ICR 7- to 8-week-old females and 8-week-old males were purchased from SPF (Beijing) Biotechnology. The ICR mice were raised under specific-pathogen-free (SPF) conditions and handled following the guidelines of the Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences (Ethical approval No. IOZ20180058).

***In vitro* culture medium.** Flush medium: CMRL 1066 (11530037, Invitrogen) + 5 × Penicillin-Streptomycin (60162ES76, YEASEN) + 10% FBS (SE200-ES, Vistech).

I: (medium on day 0-1 of IVC) CMRL 1066 (11530037, Invitrogen) + 1 × Penicillin-Streptomycin (60162ES76, Yeasen) + 1 × Gluta MAX™ Supplement (35050061, Thermo) + 1 × MEM Non-Essential Amino Acids Solution (11140050, Thermo) + 0.5 × N-2 Supplement (17502048, Gibco) + 0.5 × B-27™ Supplement (17504044, Gibco) + 10% FBS (SE200-ES, Vistech).

II: (medium on day 2 of IVC) CMRL 1066 (11530037, Invitrogen) + 1 × Penicillin-Streptomycin (60162ES76, Yeasen) + 1 × Gluta MAX™ Supplement (35050061, Thermo) + 1 × MEM Non-Essential

Amino Acids Solution (11140050, Thermo) + 0.5 × N-2 Supplement (17502048, Gibco) + 0.5 × B-27™ Supplement (17504044, Gibco) + 20% FBS (SE200-ES, Vistech).

III: (medium on day 3-4 of IVC) CMRL 1066 (11530037, Invitrogen) + 1 × Penicillin-Streptomycin (60162ES76, Yeasen) + 1 × Gluta MAX™ Supplement (35050061, Thermo) + 1 × MEM Non-Essential Amino Acids Solution (11140050, Thermo) + 30% KnockOut™ Serum Replacement (10828028, Gibco).

IV: (medium on day 5 of IVC) CMRL 1066 (11530037, Invitrogen) + 1 × Penicillin-Streptomycin (60162ES76, Yeasen) + 1 × Gluta MAX™ Supplement (35050061, Thermo) + 1 × MEM Non-Essential Amino Acids Solution (11140050, Thermo) + 30% KnockOut™ Serum Replacement (10828028, Gibco) + 30% RS.

V: (medium on day 6-10 of IVC) CMRL 1066 (11530037, Invitrogen) + 1 × Penicillin-Streptomycin (60162ES76, Yeasen) + 1 × Gluta MAX™ Supplement (35050061, Thermo) + 1 × MEM Non-Essential Amino Acids Solution (11140050, Thermo) + 50% RS[53].

U3N system. The mass ratio of matrix and crosslinker in the PDMS prepolymer (Sylgard 184, Dow Corning, United States) was 10:1. The prepolymer was blended uniformly and poured over the four-well plate (176740, Thermo). PDMS prepolymer (0.2 g) was added per well, and the PDMS wells were cured at 80°C for 2 h and were processed by plasma 30 w for 5 min. The PDMS surface was pretreated with 0.2 mg/mL sulfo-SANPAH (22589, Thermo) and was coated with 50 μg/mL type I collagen (5133, Advanced BioMatrix) for 10 h. A dialysis of type I collagen (5133, Advanced BioMatrix) was added to the coated PDMS wells, and they were cured at 37°C for 1 h.

Embryo recovery and culture. Pregnant mice were humanely culled at 3.5 days post coitum through cervical dislocation, and the embryos were flushed out with flush medium[16]. The embryos were seeded onto a specific well plate. This time was recorded as day 0 of IVC. The medium on day 2 of IVC was replaced on the evening of the second day, and a corresponding number of days was changed every day. The embryo cultures were performed at 37°C in a 5% CO_2 atmosphere.

Fixation and immunocytochemistry. Embryos were fixed overnight with 4% paraformaldehyde at 4°C; permeabilised for 1 h in 1% Triton X-100 in PBS at 37°C; blocked for 1 h in 0.1% Tween-20, 0.01% Triton X-100 and 1% BSA in PBS at 37°C; and incubated with primary antibodies at 4°C overnight. The primary antibodies were OCT4 (1:100, sc-8629, Santa Cruz), F-ACTIN (1:100, ab205, abcam), CDX2 (1:100, 3977S, Cell Signaling Technology), Collagen I (1:100, ab90395, abcam), FOXA2 (1:100, 8186S, Cell Signaling Technology) and TFAP2C (1:100, sc-12762, Santa Cruz). The secondary antibodies were incubated for 2 h at room temperature. The secondary antibodies were Alexa 488 donkey anti-mouse (1:500, A21202, Invitrogen), Cyanine3 goat anti-mouse (1:500, A10521, Invitrogen), Alexa 488 goat anti-rabbit (1:500, A11034, Invitrogen) and Cyanine3 goat anti-rabbit (1:500, A10520, Invitrogen). Nuclear staining and incubation in 10 μg/mL Hoechst 33342 (H3570, Invitrogen) were performed for 10 min at room temperature. For larger embryos (≥day 6 of IVC or ≥E6.5), the protocol described by Yang[54] was

followed. The embryos were imaged on a Leica SP8, Zeiss LSM780 confocal microscope or Zeiss Lighsheet Z.1. Imaris 9.0.2 software was used to construct the 3D images.

Embryo transfer

E4.5 embryos cultured on PDCO and PD were transferred to the oviduct of pseudo-pregnant ICR mice at E2.5. To evaluate the development of the embryos, the pregnant recipients were dissected at day 6 after transplantation.

Uterus decellularization. Uterine perfusion was performed through the aorta (flow rate = 2 mL/min) with 0.1% sodium dodecyl sulphate and 1% Triton-X100 for 12 h and then with phosphate buffered saline for 24 h, after which the decellularised uterus was fixed.

Scanning electron microscopy. At 4 °C, the decellularised uterus matrix or collagen were fixed for 4 h in 2.5% glutaraldehyde. Then the samples were dehydrated for each 15 min in 30 vol.%, 50 vol.%, 75 vol.%, 80 vol.%, 90 vol.% ethanol and 45 min in 100 vol.% ethanol. The samples after supercritical drying (aotosamdri-815, Tousimis) were sprayed with platinum (JEC-3000FC, JEOL) for SEM observation (SU8010, Hitachi).

Single-cell isolation and transcriptome library construction. Single-cell isolation and transcriptome library construction were performed according to the protocol described previously[55]. The single-cells from embryos of PD and PDCO on day 2 of IVC were harvested to generate RNA-seq data compared to the embryos of E4.5 for analysis[28]. The raw files were processed with Cell Ranger using the default mapping arguments. Gene expression analyses and cell-type identifications were performed using Seurat V3.1[56]. Our data were merged and normalised with published data using the IntegrateData function, as described in the Seurat vignettes. The tSNE was used for nonlinear dimensional reduction. The figures were produced in Seurat using the DimPlot and VlnPlot functions.

RNA-seq library preparation and data analyses. Total RNA was extracted from the collected samples with PureLink™ RNA Mini Kit (Life). The RNA-seq libraries were prepared with the NEBNext®UltraTM RNA Library Prep Kit for Illumina®. Sequencing was performed on an Illumina HiSeq X-Ten sequencer with a 150 bp paired-end sequencing reaction. The clean reads were analysed with Hisat2 (version 2.1.0) and StringTie, using the mm10 annotation and default settings48,49. Reads with unique genome locations and genes with no less than 1 FPKM in at least one sample were used for the next step of the analyses. The threshold for filtering differentially expressed genes was set at 2-fold: increased or decreased expression that was less than 2-fold was not considered significant. The tissue lineage marker genes were downloaded from https://marionilab.cruk.cam.ac.uk/MouseGastrulation2018/, heatmap analyses were performed with hierarchical cluster and the heatmap.2 functions in R. Gene ontology analyses were performed using DAVID online.

Modulus test. At least three samples were prepared and tested that included uterine or biological material. Mark-10 ESM303 (Mark-10 Corporation, NY, USA) was used to perform the tensile test and compression

test. We used the tensile test tool (Mark-10 G1008) or compression test tool (Mark-10 G1009 and GG1029) to fix the uterus or biological material. Loading was applied at a rate of 10 mm/min until a maximum force of 10 N was achieved. The load and continuous displacement were measured directly from the test stand's control panel (Mark-10 ESM 303-004), and load and displacement were recorded in real time using the MESUR gauge Plus (Mark-10 15-1005) software.

Atomic force microscopy. The mass ratio of matrix and crosslinker in the PDMS prepolymer (Sylgard 184, Dow Corning, United States) is 1:10. The prepolymer was blended uniformly and poured over the plate (351006, Falcon) until the PDMS thickness was 70 μm. The plates were cured at 80°C for 2 h and then were processed by plasma 30 w for 5 min. The PDMS surface was pretreated with 0.2 mg mL^{-1} sulfo-SANPAH (22589, Thermo) and was coated with 50 μg mL^{-1} type I collagen (5133, Advanced BioMatrix) for 10 h. The coated PDMS wells were added a dialysis of type I collagen (5133, Advanced BioMatrix), which was cured at 37°C for 1 h. E3.5 embryos were seeded on the plates and cultured to day 2 of IVC.

Sample measurement. All experiments were conducted on a Bruker Icon AFM. Bruker PFQNM-LC-A-CAL probes with a force constant of 0.1 N/m were used for all experiments, and all measurements were performed in the liquid. The modulus of cells on different materials was analysed using NanoScope.

Statistical analyses. Statistical analyses were performed in GraphPad Prism 5 and Origin 9. using Fisher's exact test, one-way ANOVA with Bonferroni multiple comparison post hoc test or two-way ANOVA with Bonferroni post hoc test. The data are represented as mean ± s.d. Statistical significance was set at $P < 0.05$.

Data availability. The sequencing data are deposited in the Genome Sequence Archive of the Beijing Institute of Genomics, Chinese Academy of Sciences (http://gsa.big.ac.cn/). The accession number for mouse bulk and single-cell RNA-Sequence data reported in this project are PRJCA004049.

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Declarations

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Author contributions

Q.G. conceived and designed the project together with Z.G., S.-T.W., and H.-M.W.; Z.G., J.G. and J.-L.Z. equally designed and performed all experiments and prepared the manuscript; G.-H.F., X.-N.W., Z.-L.G., L.-Y.J., Y.-Q.W., L.W., J.H., and W.L. provided assistance in carrying out experiments and discussed results. Z.G., J.G. and J.-L.Z., together with S.-T.W., H.-M.W., and Q.G. discussed results and prepared the manuscript.

Competing interests

The authors declare no competing interests.

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