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

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Supplementary information for

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**Supplementary notes**

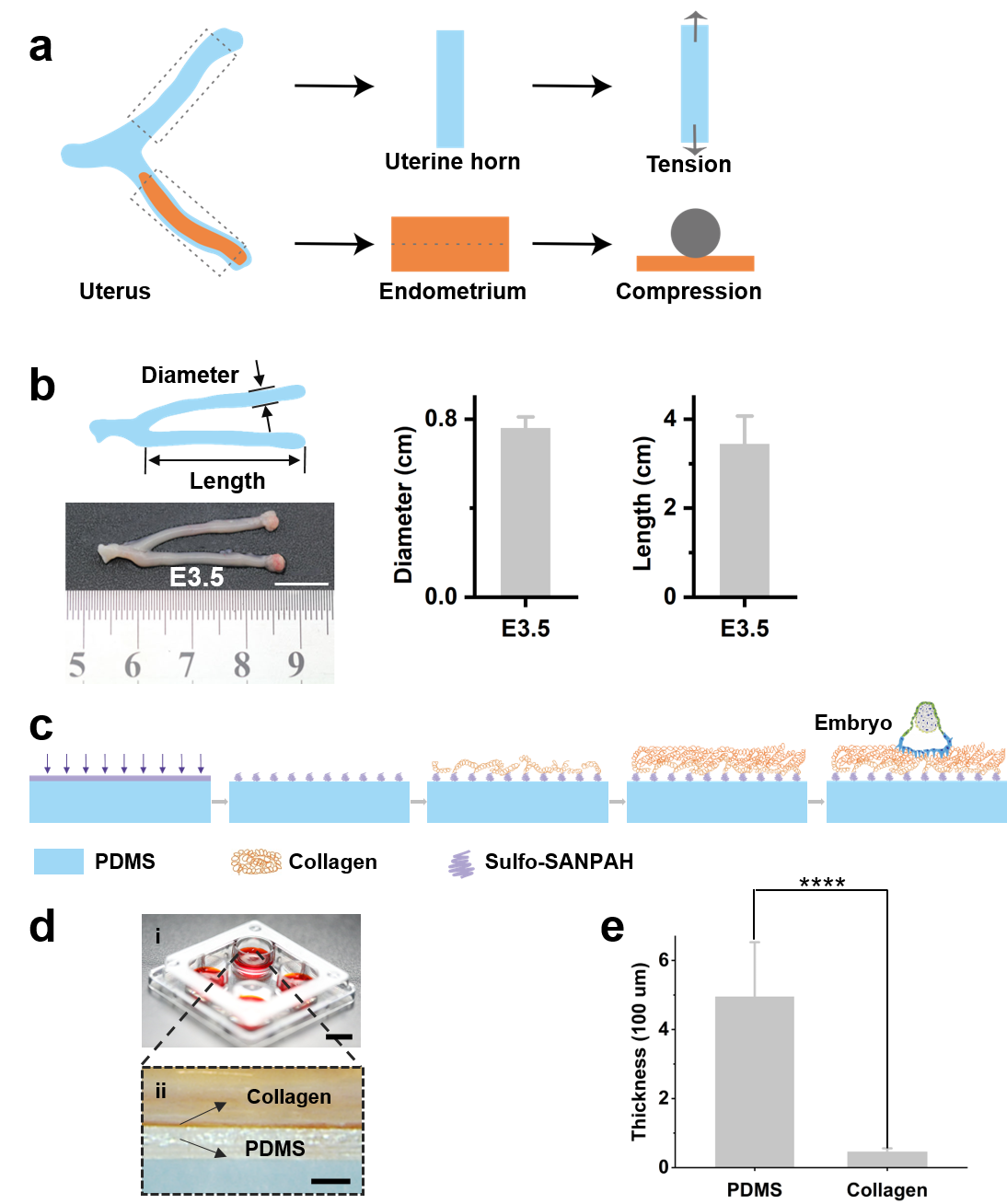
**Previous studies on embryo culture and embryo-material interactions**

The first mouse blastocyst cultured *in vitro* was injected into the fiber network of the bovine eye1. Currently, the typical dishes used for embryo culture are made of polystyrene, silicone or specialized plastic polymer-coated by collagen, laminin, fibrinogen or some other matrix2-6.

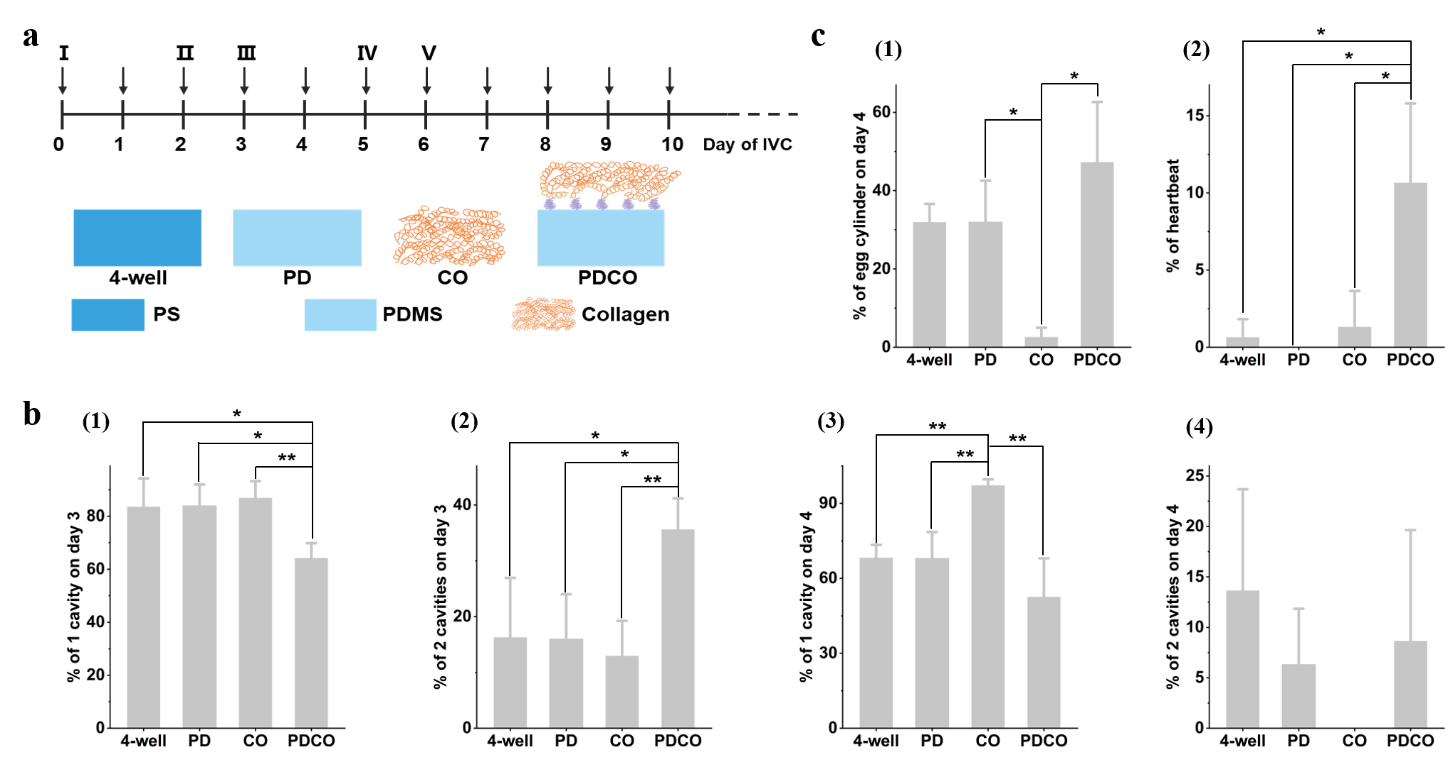
Cell microenvironment, a complicated and dynamic biochemical and physical environment, is able to control cell behaviors and development and determine cell fates7. An embryo, the well-organized cell cluster, is also sensitive to the surrounding condition8,9.

3D biomaterial system has provided a paradigm shift incell culture *in vitro* and considerable research efforts have been devoted to material properties including macro/nano-structure, elastic, stuffiness, micro/nano-patterns influencing cell states10-14 through chemical pathways15-20.

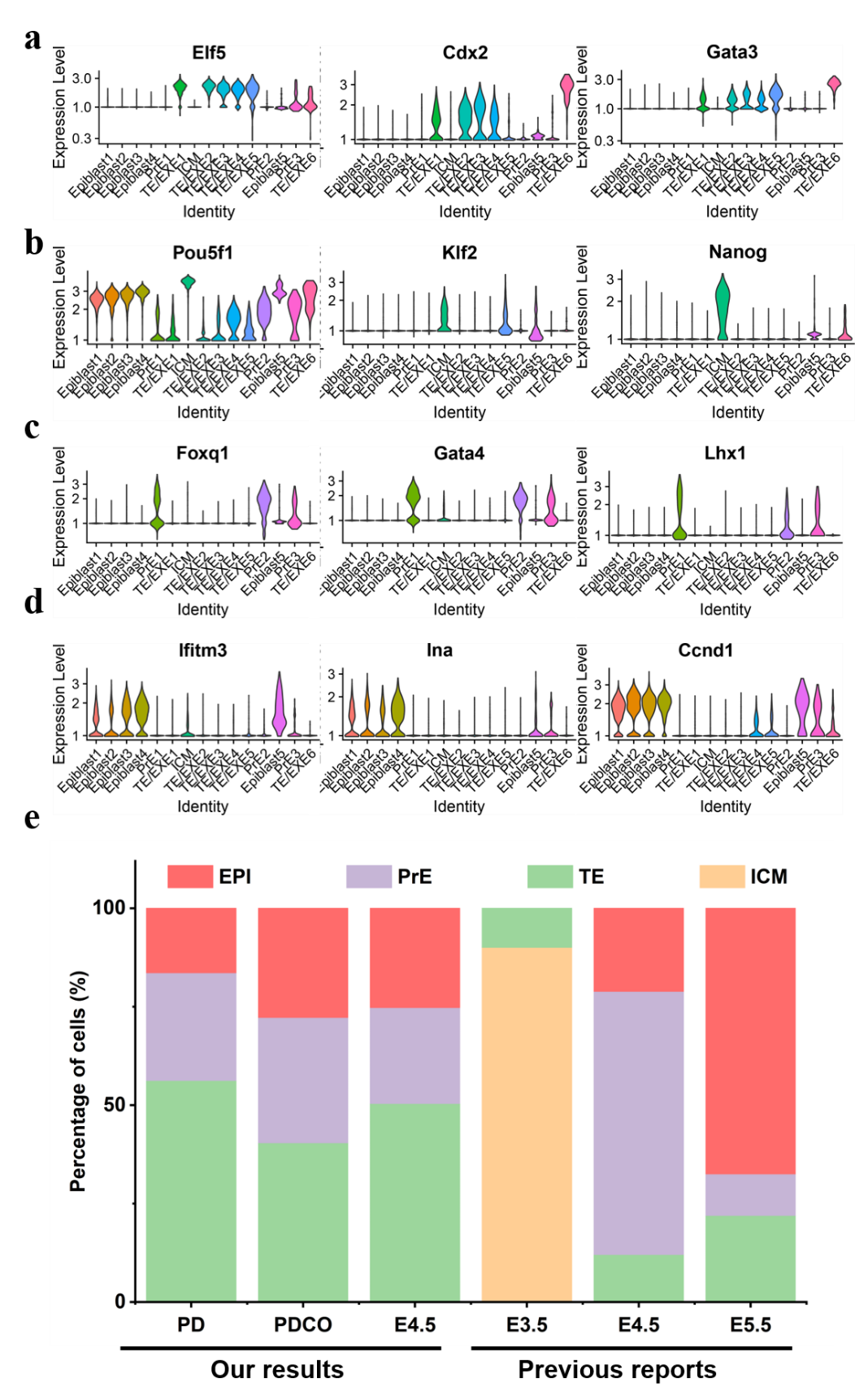
Our U3N platform was established according to the well understanding of the structure of the uterus and developmental biology. Developing of appropriate culture method in vitro is crucial to explore the black-box of embryo development, and stem cell based embryo assembly, which have attracted much attention21-30. To the best of our knowledge, U3N system not only extends the embryo culture period to heartbeat stage from initial blastocyst, but also uncovers molecular regulators between materials and embryos, which governs YAP pathways, to regulate embryo spreading and developmental potential31-36.



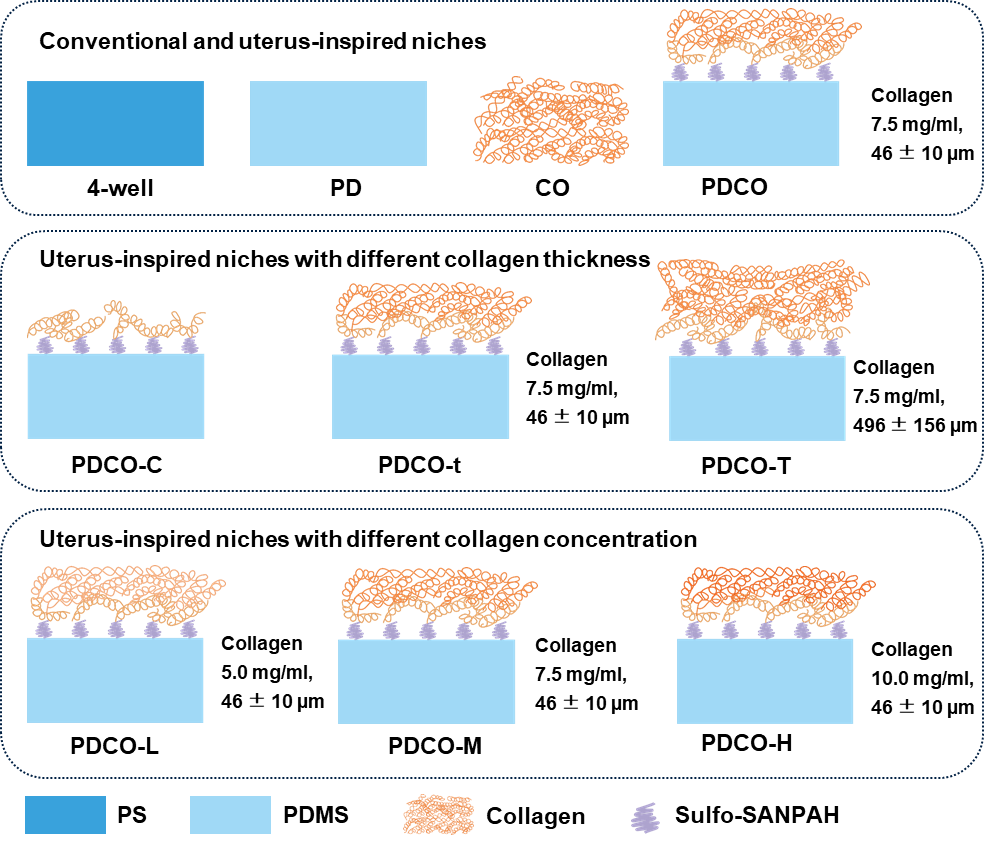
**Supplementary Figure 1** **Uterus and** **uterus-inspired 3D niche construction. a,** Schematic diagram of uterine modulus measurement. **b**, Digital morphology picture of E3.5 mouse uterus with uterine schematic in the left, statistics of diameter and length of uterine horn in the middle and right. Scale bar, 1 cm. **c**, Preparation process of uterus-inspired 3D niche. **d**, Digital image (top panel) of the in vitro culture environment for the embryo on the uterus-inspired 3D niche. Scale bar, 3 mm. Magnified view of cross section of culture substrate is shown at the bottom panel. (i) scale bar, 1 cm; (ii) Scale bar, 500 μm. **e**, The thickness of PDMS and collagen in the major area of embryo development in the dotted box (**d**). \*\*\**P* < 0.001.



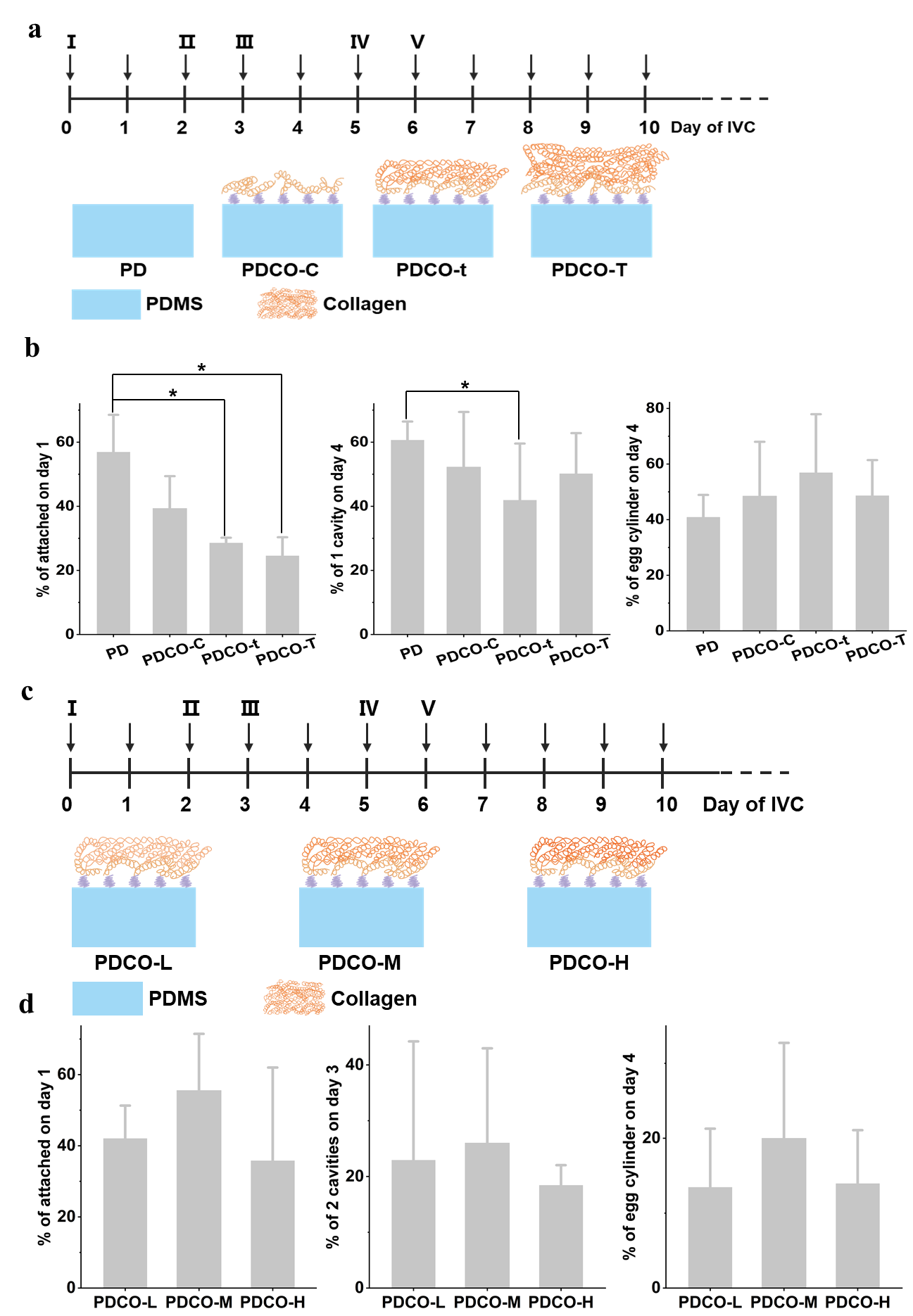
**Supplementary Figure 2** **Embryo development in conventional and** **uterus-inspired niche. a**, Schematic representation of conventional and uterus-inspired niche. **b**, **c**, Developmental status of embryos in conventional and uterus-inspired niche. I, II, III, IV and V represent the medium added at the corresponding time points of *in vitro* culture: І, the medium for embryos on day 0-1 of IVC, Ⅱ, the medium for embryos on day 2 of IVC, Ⅲ, the medium for embryos on day 3-4 of IVC, Ⅳ, the medium on day 5 of IVC, Ⅴ, the medium for embryos on day 6-10 of IVC. \**P* < 0.05, \*\**P* < 0.01.



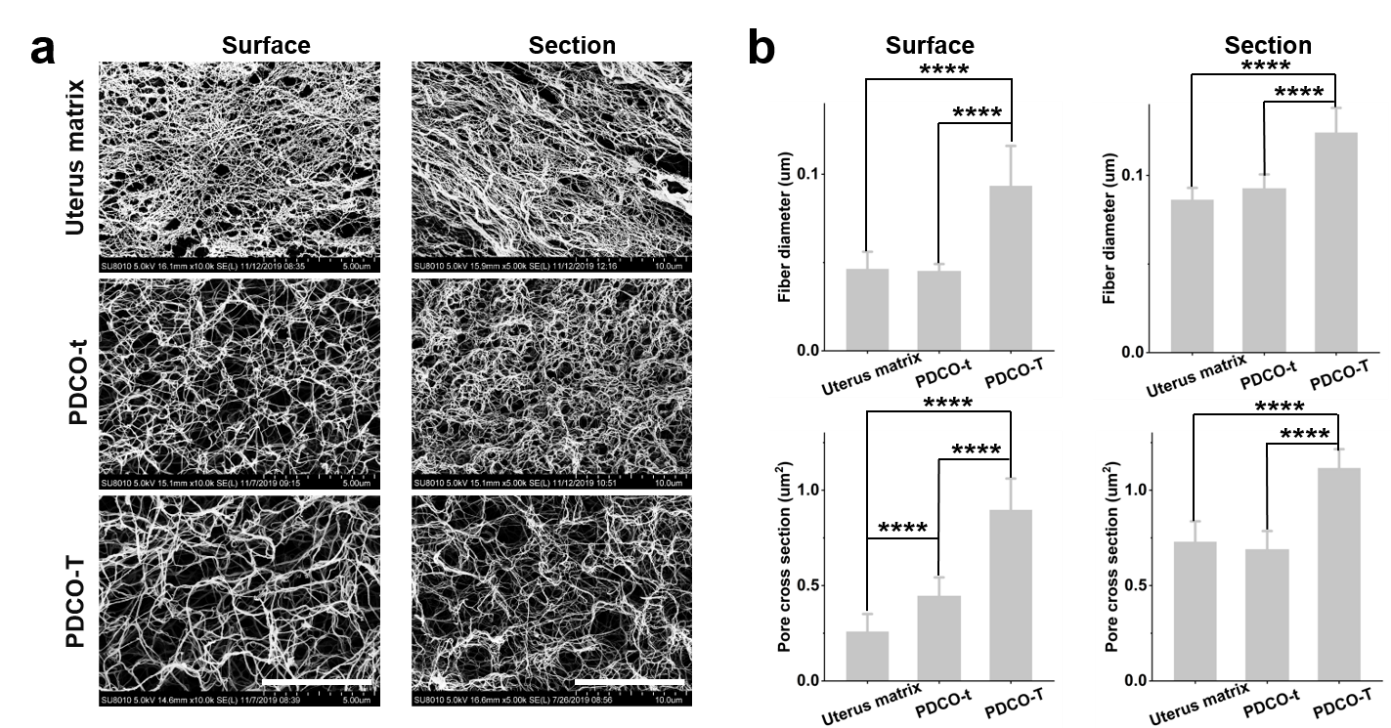
**Supplementary Figure 3** **Results of single-cell RNA sequencing.** **a-d**, Expression of clustering maker genes for TE (a), ICM (b), PrE (c), EPI (d). **e**, Proportion of EPI, PrE, TE, and ICM cells of embryos on PD and PDCO on day 2 of IVC and embryos of E3.5, E4.5 and E5.5 in previous publications. Epiblast (EPI), primitive endoderm (PrE), trophoblast (TE)and inner cell mass (ICM).



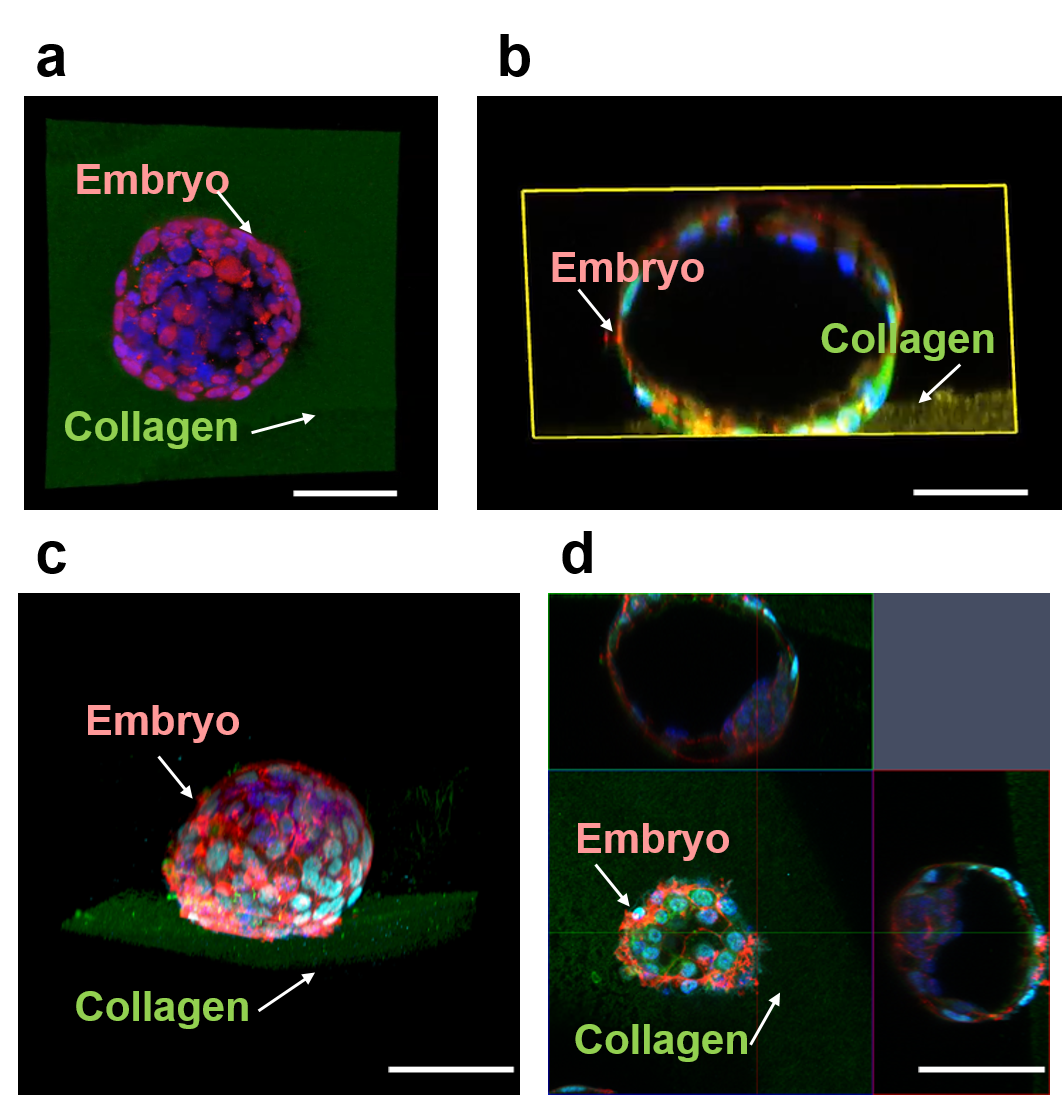
**Supplementary Figure 4** **Schematic diagram and abbreviations of major terms of the niche for embryo culture in vitro. Up panel**, the conventional (4-well and PD) and uterus-inspired niches (PDCO).4-well: Nunc 4-well dish. PD: PDMS with a thickness of 496 ± 156 µm. CO: Collagen with a concentration of 7.5 mg/ml and a thickness of 46 ± 10 µm. PDCO: The bottom layer is PDMS (496 ± 156µm in thickness) and the up layer is collagen (7.5 mg/ml, 46 ± 10 µm in thickness). **Middle panel**, different thickness of collagen gel for the uterus-inspired niche. PDCO-C: PDMS (496 ± 156µm in thickness) is coated with collagen. PDCO-t: The bottom layer is PDMS (496 ± 156µm in thickness) and the up layer is collagen (7.5 mg/ml, 46 ± 10µm in thickness). PDCO-T: The bottom layer is PDMS (496 ± 156µm in thickness) and the up layer is collagen (7.5 mg/ml, 417 ± 137µm in thickness). **Bottom panel**, different concentrations of collagen gel for the uterus-inspired niche. PDCO-L: The bottom layer is PDMS (496 ± 156µm in thickness) and the up layer is collagen (5.0 mg/ml, 46 ± 10µm in thickness). PDCO-M: The bottom layer is PDMS (496 ± 156µm in thickness) and the up layer is collagen (7.5 mg/ml, 46 ± 10µm in thickness). PDCO-H: The bottom layer is PDMS (496 ± 156µm in thickness) and the up layer is collagen (10.0 mg/ml, 46 ± 10 µm in thickness).



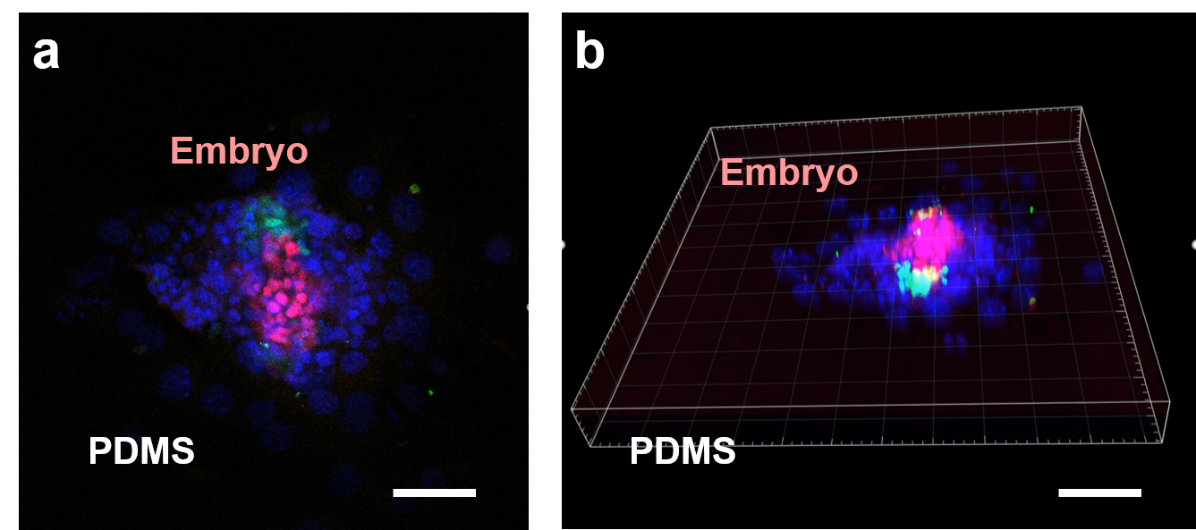
**Supplementary Figure 5** **Optimization of the uterus-inspired niche to promote embryo development. a**, **b**,The embryo development in the uterus-inspired niche with different collagen thickness. **c**, **d**, The embryo development in the uterus-inspired niche with different collagen concentration. I, II, III, IV and V represent the medium added at the corresponding time points of *in vitro* culture: І, the medium for embryos on day 0-1 of IVC, Ⅱ, the medium for embryos on day 2 of IVC, Ⅲ, the medium for embryos on day 3-4 of IVC, Ⅳ, the medium on day 5 of IVC, Ⅴ, the medium for embryos on day 6-10 of IVC. \**P* < 0.05.



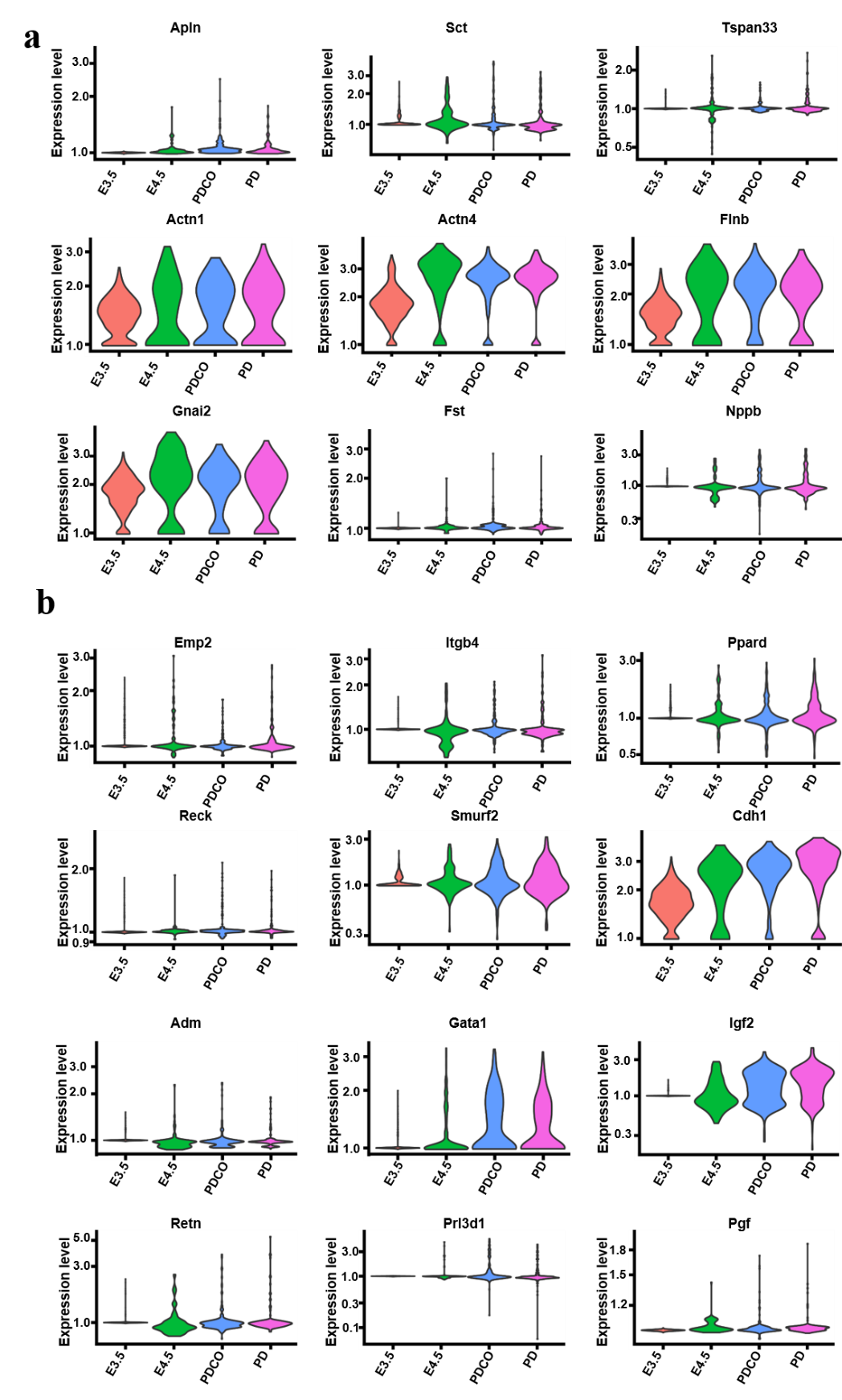
**Supplementary Figure 6** **Developmental status of embryos in the uterus-inspired niche with different collagen thickness. a**,Scanning electron microscopy images of the surface and cross sections of uterus matrix and indicated culture matrix. Scale bars, 5 μm (left panel). Scale bars, 10 μm (right panel). **b**, The fiber diameter and pore cross sections of surface (right panel) and cross sections (left panel) of uterus matrix and indicated culture matrix. \*\*\*\**P* < 0.0001.



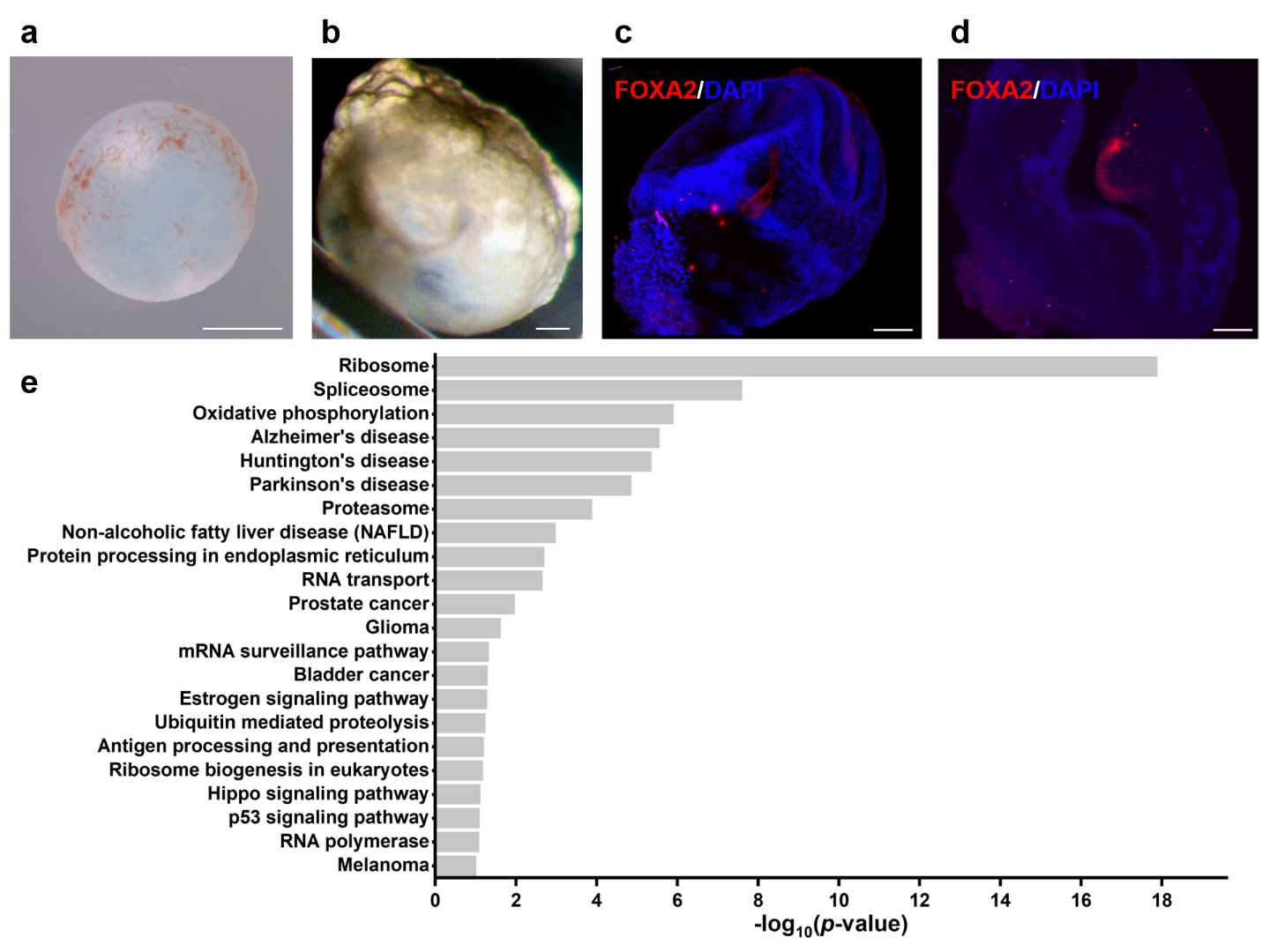
**Supplementary Figure 7** **Embryo invasion into collagen fibers. a, b**, Screenshots of 3D video of the E3.5 embryo cultured on the PDCO for one day and the cross section perpendicular to collagen substrate. n = 3. Scale bar, 100 μm. a, CDX2, red (trophoblast), and Collagen I, green (material), DAPI, blue (nucleus), b, CDX2, cyan (trophoblast), Collagen I, yellow (material), Phalloidin, red (cytoskeleton), DAPI, blue (nucleus). **c, d**, 3D images of embryos on PDCO of day 2 for IVC. CDX2, cyan (trophoblast), Collagen I, green (material), Phalloidin, red (cytoskeleton), DAPI, blue (nucleus). The above and right images are cross-sectional views along the corresponding color indicator lines n = 5. Scale bars, 100 μm.



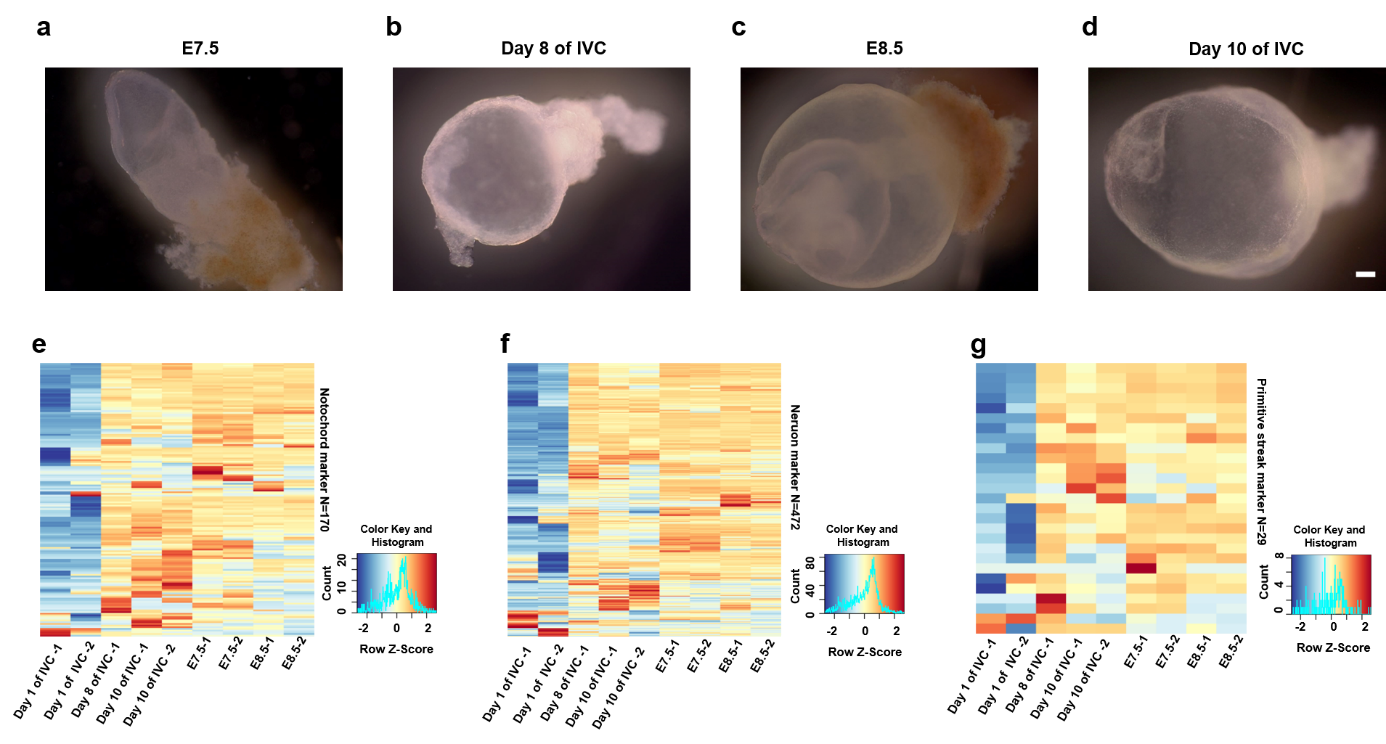
**Supplementary Figure 8 3D imaging of the embryos cultured on PD.** The video screenshots of the embryos at E3.5 cultured on the PD for one day that are displayed dynamically from top to bottom (**a**) and by rotating from multiple angles (**b**). OCT4, green (epiblast), CDX2, red (trophoblast), and DAPI, blue (nucleus). Scale bars, 100 μm.



**Supplementary Figure 9** **Detection of embryo hormones and implantation genes in vitro and in vivo.** RNA analysis markers for hormones (**a**) and implantation-related (**b**) of the embryos of 2 days of IVC on PD and PDCO and normal embryos of E3.5 and E4.5.



**Supplementary Figure 10** **Progressive development of embryos at post-gastrulation in uterus-like microenvironment.** (**a**) Morphological observation of the yolk sacs of embryo culture on PDCO at day 8. Scale bars, 1 mm. (**b**) Video screenshot of embryo culture on PDCO for 10 days with early cardiac formation and pulsation. Scale bar, 200 μm. **c**, E8.5 embryos were stained late embryonic brain with antibody for FOXA2 (red). Scale bar, 200 μm. **d**, Mouse blastocysts were cultured on PDCO until day 8, and stained late embryonic brain with antibody for FOXA2 (red)).. Scale bar, 200 μm. (**e**) Compared to embryos cultured on the PDMS, KEGG of upregulated genes of embryos cultured on the PDCO for 10 days.



**Supplementary Figure 11** **Characterization of embryos cultured at post-implantation.** (**a-d**) Bright images of embryos on day 8 and day 10 of IVC on PDCO and normal embryos of E7.5 and E8.5. Scale bars, 200 μm. (**e-g**) RNA sequencing of the embryos mentioned above and analysis for the markers of neural tissue, primitive strips, and chordae.

**Supplementary Table 1**

Compared to the embryos cultured on the PDMS, GO terms of upregulated genes in embryos on day 2 cultured on the PDCO.

|  |  |
| --- | --- |
| Terms | Genes |
| oxidation-reduction process | *ALDH18A1, NDUFB7, PYROXD1, CHCHD4, FTH1, NDUFB2, UQCR11, HIF1AN, HMOX1, PNPO, AKR7A5, CAT, ETFB, GPD2, NDUFA4, SEPW1, GDI2, PTGR1, NDUFB10, AIFM1, NDUFA7, NDUFC1, SOD1, TET1, NDUFA1, NDUFA12, NDUFA11, AKR1B8, ADI1, NDUFV3, TXNDC12, PYCR2, KDM2A, RRM2, JMJD1C* |
| negative regulation of intrinsic apoptotic signaling pathway | *G2E3, HDAC2, PARL, NDUFA13, HELLS, NOC2L* |
| cell proliferation | *MORF4L1, PTGES3, USPL1, MKI67, MAP2K1, NASP, HLCS, GRHL2, TACC2, LARP1, PURA, SRRT, DKC1, GNG2, H3F3B* |
| ATP metabolic process | *ATP5J2, ATP5O, ATP5G1, HSPA8, ATP5K, ATP5J* |
| cellular response to retinoic acid | *WNT7B, HDAC2, HTRA2, NDUFA13, SNRNP70, ABL2, PHC1* |
| progesterone receptor signaling pathway | *NEDD4, PHB, UBR5* |
| cell migration | *CORO1B, CCDC88A, S1PR1, GSK3A, EFNB2, LAMC1, ARPC5, CD63, MDK, PTEN, ABL2, LCP1* |
| in utero embryonic development | *FGFR2, MYO1E, TRIM28, GJB3, CDH1, PRKCSH, RBBP6, GRHL2, GPI1, YBX1, WNT7B, SALL4, KDM2A, ANKRD11, MEG3, SYF2* |
| multicellular organism growth | *FGFR2, GPD2, KDM2A, KMT2C, ANKRD11, MEG3, H3F3B, RBBP6, GRHL2* |
| positive regulation of gene expression | *FGFR2, PTGES3, HMGB2, MAP2K1, PHB, LEF1, CALR, HNRNPU, RPS3, DROSHA, WNT7B, ID2, KDM2A, UBR5, MDM2, HSPA4, TCF3, DNMT3B, HSPA8* |
| embryonic organ development | *FGFR2, WNT7B, SYF2, RBBP6, GRHL2* |
| positive regulation of cell cycle | *FGFR2, ID2, MDM2, CALR, TCF3* |
| embryo implantation | *GRN, FKBP4, TRIM28, CDH1, H3F3B, SOD1* |
| epithelial cell morphogenesis | *CDH1, JMJD1C, GRHL2, IFT88* |

**Supplementary Table 2**

Compared to embryos cultured on the PDMS, KEGG of upregulated genes in embryos on day 2 cultured on the PDCO

|  |  |
| --- | --- |
| Terms | Genes |
| Ribosome | *RPL17, RPL19, MRPS14, RPL14, RPL13, RPLP2, RPL37, RPL38, RPS3, RPS25, MRPL11, RPS27, RPS29, RPL8, RPL3, FAU, RPL10A, RPL7A, MRPL33, RPS27A, RPL26, RPS5, RPS18, MRPS18C, RPS19, RPL41, RPL18A, MRPL27, RPL13A, RPS14, RPL21, RPS15, RPS12, RPS11* |
| Spliceosome | *BCAS2, EFTUD2, PPIL1, LSM7, DDX39B, SNRPD2, SF3A1, HNRNPU, HNRNPM, PPIH, SRSF9, SYF2, DHX16, SLU7, ACIN1, SNRNP70, SNRNP27, THOC2, PRPF38B, HSPA8, DDX42* |
| Oxidative phosphorylation | *NDUFA4, ATP5J2, NDUFB10, NDUFB7, NDUFA7, COX7C, COX4I1, NDUFA13, ATP5G1, NDUFC1, NDUFA1, NDUFA12, NDUFB2, NDUFA11, NDUFV3, UQCR11, ATP5O, ATP5K, ATP5J* |
| Alzheimer's disease | *NDUFA4, NDUFB10, NDUFB7, NDUFA7, COX7C, COX4I1, NDUFA13, ATP5G1, NDUFC1, NDUFA1, NDUFA12, NDUFB2, CAPN1, NDUFA11, NDUFV3, UQCR11, BACE1, CALM3, ATP5O, CALM1, ATP5J* |
| Huntington's disease | *NDUFA4, POLR2F, NDUFB10, NDUFB7, POLR2J, NDUFA7, COX7C, COX4I1, NDUFA13, ATP5G1, NDUFC1, REST, SOD1, NDUFA1, NDUFA12, NDUFB2, NDUFA11, NDUFV3, UQCR11, HDAC2, ATP5O, ATP5J* |
| Parkinson's disease | *NDUFA4, NDUFB10, NDUFB7, NDUFA7, COX7C, COX4I1, NDUFA13, ATP5G1, NDUFC1, NDUFA1, NDUFA12, NDUFB2, NDUFA11, NDUFV3, UQCR11, HTRA2, ATP5O, ATP5J* |
| Proteasome | *PSMB6, PSME1, PSME2, PSMA4, PSMB3, PSMC1, PSMB2, POMP, PSME4* |
| Non-alcoholic fatty liver disease (NAFLD) | *NDUFA4, NDUFB10, NDUFB7, NDUFA7, COX7C, COX4I1, NDUFA13, NDUFC1, NDUFA1, NDUFA12, NDUFB2, NDUFA11, NDUFV3, UQCR11, GSK3A* |
| Protein processing in endoplasmic reticulum | *HSP90AB1, GANAB, PDIA3, RRBP1, UBE4B, PDIA4, CALR, PRKCSH, SSR1, RBX1, CAPN1, HSP90B1, ATF6B, DNAJA1, HSPA8* |
| RNA transport | *EEF1A1, XPO1, EIF2S3Y, DDX39B, EIF5B, RANGAP1, EIF4G1, EIF3A, EIF4EBP1, UPF3B, RAE1, EIF1, PABPC1, ACIN1, THOC2* |
| Prostate cancer | *HSP90AB1, FGFR2, CCNE2, HSP90B1, MAP2K1, PDGFA, MDM2, LEF1, PTEN* |
| Glioma | *MAP2K1, PDGFA, CALM3, MDM2, CDK4, PTEN, CALM1* |
| mRNA surveillance pathway | *PPP1CA, UPF3B, GSPT1, DDX39B, PABPC1, ACIN1, PPP2R2D, CPSF1* |
| Bladder cancer | *MAP2K1, RASSF1, MDM2, CDH1, CDK4* |
| Estrogen signaling pathway | *HSP90AB1, HSP90B1, MAP2K1, FKBP4, ATF6B, CALM3, HSPA8, CALM1* |
| Ubiquitin mediated proteolysis | *NEDD4, UBE3A, UBE2K, UBR5, UBE4B, UBE2M, MDM2, SAE1, UBE3C, RBX1* |
| Antigen processing and presentation | *HSP90AB1, PSME1, PDIA3, PSME2, HSPA4, CALR, HSPA8* |
| Ribosome biogenesis in eukaryotes | *DROSHA, NOL6, XPO1, DKC1, NOP58, GNL3L, NOP56* |
| Hippo signaling pathway | *AJUBA, ACTB, WNT7B, PPP1CA, FRMD6, ID2, RASSF1, LEF1, CDH1, PPP2R2D* |
| p53 signaling pathway | *CCNE2, RRM2, MDM2, CDK4, PERP, PTEN* |
| RNA polymerase | *POLR2F, POLR3K, POLR2J, POLR3D* |
| Melanoma | *MAP2K1, PDGFA, MDM2, CDH1, CDK4, PTEN* |

**Supplementary movie legends**

**Supplementary movie 1**

**The embryo was viewed using confocal microscope and 3D-reconstruction of Imaris 9.0.2 software.**

Three embryos from three independent experiments displayed similar results, penetration of the E3.5 embryo cultured on PDCO for one day through the collagen fibers. a, c, d CDX2, red (trophoblast), and Collagen I, green (material), DAPI, blue (nucleus), b, CDX2, cyan (trophoblast), Collagen I, yellow (material), Phalloidin, red (cytoskeleton), DAPI, blue (nucleus). Scale bars, 100 μm.

**Supplementary movie 2**

**The embryo was viewed using confocal microscope and 3D-reconstruction of Imaris 9.0.2 software.**

Three embryos from three independent experiments displayed similar results. The video of the E3.5 embryos cultured on the PD for one day. OCT4, green (epiblast), CDX2, red (trophoblast), and DAPI, blue (nucleus). Scale bar, 100 μm.

**Supplementary movie 3**

**Original heart was developed on Day 8 of *in vitro* culture, and began to beat more than 2 days.**

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