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Supplementary information

Ex utero mouse embryogenesis from pre-gastrulation to late organogenesis

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Supplementary Information

Ex utero Mouse Embryogenesis from Pre-Gastrulation to Late Organogenesis

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SI guide

Supplementary Video Legends

Video 1. *Ex utero* mouse embryogenesis set-up. Part 1- Roller culture incubator with customized gas concentration and pressure regulation module for growing mouse embryos *ex utero*. Part 2- Media bottle exchange and transfer of an embryo into the roller culture system. Part 3- Setting up the roller culture incubator and gas regulation module.

Video 2. Late-gastrulation to organogenesis *ex utero* roller culture. Representative snapshot bright field videos of E7.5 *ex utero* grown embryos after 1-4 days of culture.

Video 3. Live embryo inside the culture bottle. Representative video of an embryo cultured in rotating bottles as seen by the naked eye.

Video 4. Comparative light sheet 3D reconstruction of *ex utero* and *in utero* embryos. E7.5 embryos grown 4 days *ex utero* and developmentally matched *in utero* embryos immunostained for Otx2/MHC-II, Sox2, and Tuj1/Sox9.

Video 5. Robust *in toto* live imaging from gastrulation to somitogenesis. Representative confocal live imaging for 58 hours of an *ex utero*-grown tdTomato^{+} embryo from early gastrulation (E6.5) to somitogenesis (E8.5).

Video 6. Early-gastrulation to organogenesis *ex utero* embryo culture. Representative bright field snapshot videos of E6.5 *ex utero* grown embryos after 1-5 days of culture.

Video 7. Pre-gastrulation to organogenesis *ex utero* embryo culture. Representative bright field snapshot videos of E5.5 *ex utero* grown embryos after 1-6 days of culture.

Video 8. Imaging neural tube closure in cultured embryos. Long-term *in toto* live imaging of neural tube closure in cultured embryos. E7.5 tdTomato^{+} mouse embryo cultured *ex utero* until E9.0 and subjected to live confocal imaging.

Supplementary Methods

Morphological evaluation of mouse embryo development

Assessment of appropriate embryo development was performed based on previously established morphological features[1,2]. Only embryos presenting all of the following features were considered as properly developed: Culture day 1 (E8.5): >4 somites, embryo curved dorsally, amnion and yolk sac are enclosing the embryo, the allantois extended into the exocoelom and started to fuse with the chorion, the circulatory system differentiated and blood circulated through the vessels encircling the yolk sac and in the embryo, beating horseshoe-like heart rudiment and foregut pocket visible in the frontal part of the embryo, closing but unfused neural folds. Culture day 2 (E9.5): >20 somites, forelimb buds clearly present, axial turning of the embryo leading the dorsal part to face outside (C-shaped embryo); establishment of the umbilical cord connected to the placental cone, plexus of yolk sac blood vessels observed, three-chambered heart, posterior neuropore closing with small opening remaining, cranial part of the neural tube closed, brain regionalized into forebrain, midbrain and hindbrain, otic pit present and separated from the epidermis, the maxillary process, mandibular and hyoid branchial arches are visible, development of the optic vesicle. Culture day 3 (E10.5): >33 somites, tail bud and hindlimb buds clearly present, paddle-shaped forelimbs, posterior neuropore closed, visible division between telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon to form a five-vesicles brain, four-chambered heart, invaginating optic vesicle, olfactory plate formed, vessels of the yolk sac form a hierarchical network of large and small-caliber vessels with red blood cells circulating around the yolk sac and the body of the embryo, formation of the fourth branchial arch. Culture +day 4 (E11): at this stage the embryos display all the features assessed at E10.5 plus developed nasal pits, invagination and closure of the lens vesicle, and paddle-shaped hindlimbs. For calculating efficiency of *ex utero* culture throughout the manuscript, the total number of embryos assessed per condition in every sampled timepoint is indicated. Embryos dissected, fixed or moved to other conditions at any point during the time-course, are subtracted from the total where relevant.

Proper development at the morphological level from E6.5 to E8.5 was assessed as follows: At E6.5, the embryos are constituted by three cell lineages: the cup-shaped pluripotent epiblast (Epi) and two extra-embryonic lineages, the extraembryonic ectoderm (ExE) and the visceral

endoderm (VE). The cavities in the embryonic and extraembryonic compartments are unified to form the pro-amniotic cavity, radial symmetry is broken in the epiblast to initiate specification of the primitive streak. After 1 or 2 days of culture *ex utero* (depending on the time of culture initiation, E5.5 or E6.5), the embryos reach the neural plate stage equivalent to E7.5, with no allantoic bud or early bud. The amniotic folds fuse to form the amnion, and the chorion develops from the ExE and the extraembryonic mesoderm. These events generate three cavities in the embryo: amniotic, exocoelomic and ectoplacental cavities. A small allantois bud is present in some of the embryos, at the base of the primitive streak, and the anterior ectoderm begins to form the future neural groove. On the following day, cultured embryos present between 4 to 8 pairs of somites with the embryo curved dorsally, the yolk sac and yolk sac blood circulation has been established, the allantois started to fuse with the chorion, head folds are well-formed as well as the invaginating foregut and beating heart.

Assessment of embryo length

Morphometric measurements were performed using the CellSens Entry 1.18 software (Olympus) by using the images of the embryos acquired every day. Length of the antero-posterior axis was measured for embryos between E6.5 to E8.5. The crown-rump length (the longest straight line from the cranial to the caudal end of the body) was measured for embryos at later stages (E9.5 to E11) after removing the yolk sac. Length of cultured embryos was compared with freshly dissected *in utero* embryos at matched embryonic stages.

Culture of mouse naive and primed embryonic stem cell lines

For generation of Epiblast Stem cells (EpiSCs), V6.5 CAGGS-EGFP cells were cultured on mouse embryonic fibroblast (MEF) feeder cells for more than 5 passages under standard EpiSC conditions as previously described[3]. Before injection, EpiSC lines were cultured on Matrigel for 1-3 passages and treated with 10μM ROCKi (Y-27632) the day before. For naïve conditions, parental CAGGS-EGFP ES cells were passaged in standard N2B27 2i/LIF conditions[3]. For generation of Epiblast-like Stem Cells (EpiLCs), CAGGS-EGFP V6.5 naïve 2i/LIF ES cells were transferred for 48h into priming medium + 1% KSR on Matrigel. Formative EpiLC state was validated by PGCLC induction competence as previously described[4]. Cells were prepared for injection by digestion with trypsin/EDTA 0.25% for 5 min followed by dilution in FBS-containing

DMEM, washed twice in 1x PBS and filtered through a 70 μm cell strainer. Finally, cells were suspended in the respective media. Cell lines were routinely checked for Mycoplasma contaminations every month (Lonza MycoAlert Kit), and all samples analyzed in this study were not contaminated.

Whole embryo electroporation

Mouse E7.5 embryos were cultured for 24 hours and subsequently microinjected with 0.1-0.5 μl of PCAGs-EGFP plasmid into the neural tube. For this purpose, individual embryos were transferred from the roller culture to a plate filled with dissection media pre-heated at 37ºC, and then hold in parallel along its antero-posterior axis with forceps and injected using a micromanipulator, taking care that the yolk sac remains intact after injection. Plasmid was diluted at 1 μg/μl in PBS and mixed with 1% fast green dye (1 mg/ml) (Sigma; F7258). Immediately after injection the embryo was transferred into PBS and electroporated using a NEPA21 super electroporator equipped with round platinum plate electrode tweezers (NepaGene). A range of voltages were tested (10-30V) to optimize the conditions that allow good plasmid integration efficiency and embryo viability after electroporation. The most optimal conditions were as follows: Two poring pulses applied at 20V with a duration of 30 milliseconds (ms) each, a pulse interval of 450ms and a decay rate of 10%, followed by five transfer pulses applied at 15V for 50ms each with an interval of 450ms between pulses and a voltage decay of 40%. Electroporated embryos were cultured for additional one to three days. Total number of reporter-expressing cells was measured using the cell counter Plugin in Fiji/ImageJ.

Lentiviral transduction of *ex utero* embryos

For the generation of lentivirus, HEK293T cells (ATCC – CRL1573) were plated in 10 ml DMEM, containing 10% FBS and Pen/Strep in 10 cm dishes, in aliquots of 3 million cells per plate. On the next day, cells were transfected with the third generation Addgene lentivirus vectors (0.8 μg of pRSV-Rev (Addgene 12253), 0.8 μg of pMDLg/pRRE (Addgene 12251), 1.6 μg of pMD2.G (Addgene 12259), using jetPEI™ transfection reagent, along with 16 μg of the target plasmid FUGW. (The pRSV-Rev and pMDLg/pRRE are the packaging vectors and pMD2.G is the envelope plasmid). The medium was replaced after 6 hr of transfection. The supernatant containing the virus was collected 48hr and 72hr following transfection, filtered using 0.45 μm

filter and concentrated by ultracentrifugation for 2hr at 25,000 rpm (RCF avg: 82,705; RCF max: 112,700). The final viral pellet was resuspended with cold PBS. For lentiviral transduction, embryos were dissected at E6.5 and transferred to a new plate filled with dissection media pre-heated at 37°C. The injection needle was mounted on a mouth pipette and filled by aspiration with concentrated lentiviral vector FUGW (titer was estimated to be 2-3 × 10^9 TU/ml). Lentiviruses were delivered by microinjection of 0.1µl fresh lentiviral solution into the amniotic cavity. Subsequently the embryos were transferred to EUCM and cultured for up to 5 days according to the protocol described above.

Generation of post-implantation intraspecies chimeric embryos by microinjection/graft and *ex utero* culture

Embryos were dissected from pregnant female mice at E7.5 and microinjected in pre-warmed dissection media with either mouse *in vitro* EpiSCs/EpiLCs (for cell culture conditions see Supplementary Information), or with cells directly transplanted from the epiblast of developmentally matched embryos. For EpiSCs and EpiLCs, Y27632 10 µM (Axon Medchem) was applied the night before injection. Cell clumps (10-25 cells) were manually detached from the plate with a pipette tip and injected into the posterior part of the epiblast with a mouth pipette by using a flat-tip microinjection needle. For efficient incorporation of the injected cells into the epiblast of the recipient embryo, cells were expelled carefully from the needle as the micropipette was drawn out of the embryo. For transplantation experiments, clumps of 10-25 cells were cut with a tungsten filament from the epiblast of E7.5 embryos that express td-Tomato ubiquitously (ICR females crossed with Gt(ROSA)26Sor$^{(CAG\text{-}tdTomato)Hze}$/*Stra8-iCre*), and immediately grafted orthotopically by using a flat microinjection needle mounted on a mouth pipette. tdTomato negative embryos from the same litter and ICRxBDF1 matched embryos were used as recipients of the graft. Afterwards, injected embryos were cultured in rotating bottles for up to four days according to the protocol described for E7.5 embryos. Total number of integrated cells was measured using the cell counter Plugin in Fiji/ImageJ.

RNA extraction and qPCR of mouse PSC lines

EpiSCs and EpiLCs lines were characterized by real-time PCR. Briefly, total RNA was isolated using Trizol (Ambion Life Technologies), and 1 µg of total RNA was reverse transcribed using

High-Capacity Reverse Transcription Kit (Applied Biosystems). Quantitative PCR analysis was performed with the SYBR™ Green PCR Master Mix (Applied Biosystems) using 10 ng of cDNA per reaction in a Viia7 platform (Applied Biosystems). Fold change was normalized to *Gapdh* expression. As expected Nanog was decreased upon priming, while Otx2 and Fgf5 primed makers were induced in both types of primed samples. T was induced in primed, but not formative EpiLC samples, as recently described[4]. The following primers were used:

Gapdh-Forward: AGTCAAGGCCGAGAATGGGAAG

Gapdh-Reverse: AAGCAGTTGGTGGTGCAGGATG

Oct4-Forward: AGAGGATCACCTTGGGGTACA

Oct4-Reverse: CGAAGCGACAGATGGTGGTC

Nanog-Forward: CTCAAGTCCTGAGGCTGACA

Nanog-Reverse: TGAAACCTGTCCTTGAGTGC

Sox2-Forward: TAGAGCTAGACTCCGGGCGATGA

Sox2-Reverse: TTGCCTTAAACAAGACCACGAAA

Klf4-Forward: GCACACCTGCGAACTCACAC

Klf4-Reverse: CCGTCCCAGTCACAGTGGTAA

Cdx2-Forward: GCGAAACCTGTGCGAGTGGATG

Cdx2-Reverse: CGGTATTTGTCTTTTGTCCTGGTTTTCA

Gata4-Forward: CACAAGATGAACGGCATCAACC

Gata4-Reverse: CAGCGTGGTGGTAGTCTG

Gata6-Forward: CTTGCGGGCTCTATATGAAACTCCAT

Gata6-Reverse: TAGAAGAAGAGGAAGTAGGAGTCATAGGGACA

Brachyury(T)-Forward: CTGTGACTGCCTACCAGAATGAGGAG

Brachyury(T)-Reverse: GGTCGTTTCTTTCTTTGGCATCAAG

Otx2-Forward: CTTCGGGTATGGACTTGCTG

Otx2-Reverse: CCTCATGAAGATGTCTGGGTAC

Fgf5-Forward: CAAAGTCAATGGCTCCCACGAAG

Fgf5-Reverse: CTACAATCCCCTGAGACACAGCAAATA.

Antibody dilutions for whole-mount immunostaining (E5.5-E8.5)

Rabbit monoclonal anti-Brachyury (D2Z3J) (Cell Signaling, 81694) 1:100; Rabbit polyclonal anti-Cdx2 (Cell Signaling, 3977) 1:100; Mouse monoclonal anti-Cdx2 (Biogenex, MU392A-UC) 1:100; Goat polyclonal anti-Gata4 (Santa Cruz, SC-1237) 1:100; Rabbit polyclonal anti-Gata4 (Abcam, Ab84593) 1:100; Rabbit monoclonal anti-Foxa2 (EPR4466) (Abcam, Ab108422) 1:100; Mouse monoclonal anti-Myosin Heavy Chain II (MF-20) (R&D, MAB4470) 1:100; Goat polyclonal anti-Otx2 (R&D, AF1979) 1:200; Rabbit polyclonal anti-Pax6 (Covance, PBR-278P) 1:100; Goat polyclonal anti-Sox2 (R&D, AF2018) 1:200; Rabbit polyclonal anti-Sox9 (Millipore, AB5535) 1:100; Goat polyclonal anti-Sox17 (R&D, AF1924) 1:100; Mouse monoclonal anti-Tubulin ß3 (Tuj1) (Covance, MMS-435P) 1:200; Chicken polyclonal anti-GFP (Abcam, Ab13970) 1:250; Mouse monoclonal anti-Oct4 (C-10) (Santa Cruz, SC-5279) 1:100; Goat polyclonal anti-Lefty 1 (R&D, AF746) 1:100, Goat polyclonal anti-mCherry/Tomato (SiCGEN, AB0040-200) 1:200.

Antibody dilutions for IDISCO (E9.5-E11.5)

Rabbit monoclonal anti-Brachyury (D2Z3J) (Cell Signaling, 81694) 1:100; Rabbit polyclonal anti-Cdx2 (Cell Signaling, 3977) 1:100; Mouse monoclonal anti-Cdx2 (Biogenex, MU392A-UC) 1:100; Goat polyclonal anti-Gata4 (Santa Cruz, SC-1237) 1:100; Rabbit polyclonal anti-Gata4 (Abcam, Ab84593) 1:100; Rabbit polyclonal anti-Foxa2 (Abcam, Ab40874) 1:50; Mouse monoclonal anti-Myosin Heavy Chain II (MF-20) (R&D, MAB4470) 1:100; Goat polyclonal anti-Otx2 (R&D, AF1979) 1:200; Rabbit polyclonal anti-Pax6 (Covance, PBR-278P) 1:100; Goat polyclonal anti-Sox2 (R&D, AF2018) 1:200; Rabbit polyclonal anti-Sox9 (Millipore, AB5535) 1:100; Goat polyclonal anti-Sox17 (R&D, AF1924) 1:50; Mouse monoclonal anti-Tubulin ß3 (Tuj1) Tuj1 (Covance, MMS-435P) 1:200; Chicken polyclonal anti-GFP (Abcam, Ab13970) 1:250, Goat polyclonal anti-mCherry/Tomato (SiCGEN, AB0040-200) 1:200; Rabbit polyclonal anti-human TEMEM119 (Invitrogen, PA562505) 1:100; Rabbit anti-hNUMA (Abcam, ab84680) 1:100.

Immunohistochemistry

For OCT-staining, embryos were fixed overnight in 4% PFA at 4°C, washed three times in PBS for 10 min each and submerged first in 15% Sucrose/PBS and then 30% Sucrose o/n at 4°C. The day after, samples were subjected to increasing gradient of OCT concentration in Sucrose/PBS

followed by embedding in OCT on dry ice and stored at - 80°C until further processing. Cryoblocks were cut with LEICA CM1950 and washed once with 1xPBS and incubated with 0.3% H_2O_2 for 20 min. After permeabilization with 0.1% Triton X-100 in PBS for 10 min., slides were again washed three times with 1xPBS for 2 min. each and blocked in 10% normal donkey serum in PBS in humidified chamber for 20 min. at RT. Slides were then incubated with proper primary antibody diluted in antibody solution (1% BSA in 0.1% Triton X-100) at 4 °C overnight. Sections were then washed three times (5 min each) in 0.1% Triton X-100 in PBS, incubated with appropriate secondary antibodies diluted in antibody solution at RT for 1 h in the dark, counterstained with DAPI for 20 min and mounted with Shandon Immuno-Mount (Thermo Scientific, 9990412). The primary antibodies used were the following: Goat polyclonal anti-Gata4 (Santa Cruz, SC-1237) 1:200; Rabbit monoclonal anti-Foxa2 (Abcam, Ab108422) 1:200.

Confocal microscopy

Whole-mount immunofluorescence and iDISCO images were acquired with a Zeiss LSM 700 inverted confocal microscope (Carl Zeiss) equipped with 405nm, 488nm, 555nm and 635nm solid state lasers, using a Plan-Apochromat 20× air objective (numerical aperture 0.8) for E5.5/E6.5 embryos, and an EC Plan Neofluar 10× air objective (numerical aperture 0.3) for E7.5 to E11.5 embryos. Images were acquired at 1024×1024 resolution. All images were acquired within the following range of parameters: Laser power: 405 nm: 10-20%; 488 nm: 5-20%; 555nm 10-40%; 635 nm: 30-80%. Gain ranged from 350 to 600. Pixel size was 1.25 µm with a z-step of 15 µm when using the 10× objective, or 0.5 µm with z-step of 5 µm when using the 20× objective. For confocal imaging, iDISCO cleared embryos were mounted in 35mm glass bottom dishes (In Vitro Scientific, D35201.5N), employing ethyl cinnamate (Sigma, 112372) as imaging solution. For chimeric embryos, all parameters during image acquisition were compared to stained non-injected control embryos, imaged with equal parameters as the injected embryos. Images and maximum intensity projections were processed using Zen 2 blue edition software 2011 (Carl Zeiss) and Adobe Photoshop CS4.

Light-sheet microscopy

3D images of cleared embryos were acquired on a light-sheet microscope (Ultramicroscope II, LaVision Biotec) operated by the ImspectorPro software (LaVision BioTec), equipped with an

Andor Neo sCMOS camera (2,560 × 2,160, pixel size 6.5 µm x 6.5 µm) 16 bit, and an infinity corrected setup 4X objective lens: LVBT 4X UM2-BG (LVMI-Fluor 4X/0.3 Mag. 4x; NA: 0.3; WD: 5.6-6.0 mm), with an adjustable refractive index collar set to the refractive index of DBE (1.56). The light sheet was generated by scanning a supercontinuum white light laser (emission 460 nm – 800 nm, 1 mW/nm – 3 (NKT photonics). The following excitation band pass filters were used: 470/40 nm for Alexa Fluor 488, 560/40nm for Rhodamine Red-X, and 617/83nm for Alexa Fluor 647. The light sheet was used at 80% width and maximum NA (0.154). Laser power ranged between 40 to 80%. The emission filters used were: 525/50 for Alexa Fluor-488, 630\75 for Rhodamine Red-X and 690/50 for Alexa Fluor-647. Stacks were acquired using 5 µm step-size and a 200ms exposure time per step. Imaris (Bitplane) was used to create 3D reconstructions and animations of the imaged embryos.

***In toto* confocal live imaging**

Live E6.5 embryos were dissected as described above and selected for tdTomato expression. Afterwards, embryos were mounted into a droplet of EUCM adhering the ectoplacental cone to the edge of a paper filter (Millipore, AABG01300) attached to a coverslip with vacuum grease. For imaging of neural tube closure, E7.5 embryos were cultured until E9.0, Td-Tomato^{+} embryos at the proper stage were chosen and moved on a droplet of culture media on a paper filter attached to a coverslip. Then, the dorsal anterior part of the embryo was exposed by opening the yolk sac, and the embryos were anchored onto the paper filter by pressing the yolk sac to the filter. After mounting the embryos, a glass-bottomed dish (MatTek, P35G-1.5-20-C) was placed on top of the embryos using vacuum grease drops on the corners of the coverslip for spacing. Subsequently, the dish was carefully inverted, filled with 2 mL of EUCM and placed in a heat- and humidity-controlled imaging chamber (37°C; 21% O_2, 5% CO_2) of an inverted Zeiss LSM700 confocal microscope. E6.5 to E8.5 imaging was performed using the 405nm and 555nm lasers and an EC Plan Neofluar 10× air objective (numerical aperture 0.3) with 0.5× digital zoom out and a resolution of 512×512 pixels. Laser power was 1% for the 405 nm laser and 8% for the 555nm laser. Gain ranged from 500 to 600. Pixel size was 2.5 µm with a z-step of 25 µm. E9.0 embryos were imaged using the 555nm laser (8% power) and an EC Plan Neofluar 5× air objective (numerical aperture 0.16) with 0.5× digital zoom out and a resolution of 512×512 pixels. Pixel size was 2.5 µm with a z-step of 50 µm. Time interval was 1 hour for E6.5 to E8.5 embryos, and 15

minutes for E9.0. Movies were processed using Zen 2 blue edition software 2011 (Carl Zeiss) and Fiji/ImageJ.

Mouse blastocyst micromanipulation and culture of chimeric embryos

Mouse naïve V6.5 CAGGS-EGFP ES cells expanded in 2i/LIF conditions were injected into BDF2 diploid blastocysts as previously described[5]. Ten to fifteen injected blastocysts were transferred to each uterine horn of 2.5 d.p.c pseudo-pregnant females. At E7.5 chimeric embryos were dissected out of the maternal uterus and grown in the roller culture for up to three days using the same conditions described for E7.5 embryos.

***In vitro* derivation of microglial precursors from human ESCs and generation of human-mouse interspecies chimeric embryos**

WIS2 human ESCs were cultured in 5% O_2 on mTeSR1 (Stem cell Technologies) on Matrigel-coated plates. Cultures were passaged every 5–7 days by using TrypLE (GIBCO). ROCK inhibitor (Y-27632, 5-10μM) was added 24 hours before and after cell passaging. A CAGGS-EGFP reporter was introduced into the primed human ESC cells by electroporation. Microglia differentiation was performed according to Wilgenburg et.al.[6] Briefly, a suspension of $10x10^5$ human ES cells per mL was prepared on embryoid body (EB) formation media consisting on mTESR1, 50ng/ml human VEGF, 50ng/ml BMP4, 20ng/ml human SCF and 10 μM Y-27632. Embryoid bodies were generated by centrifugation on U-shaped bottom Nunclon Sphera 96-well plates (ThermoFisher Scientific, 174925). Medium was refreshed every two days. At day four, EBs were transferred to 6-well plates on differentiation medium, consisting on X-VIVO 15 media (Lonza, BE02-060F) supplemented with IL-3 (25ng/ml), M-CSF (100ng/ml) penicillin/streptomycin (100UI/ml), Glutamax 1X, and 50 μM ß-mercaptoethanol. Media was exchanged every week and cells in the supernatant started to be harvested after three weeks. Characterization and validation of human ESCs-derived microglial precursors was performed by flow cytometry on a BD FACS-Aria III. Cells were incubated for half an hour with CD34-PE (BioLegend, 561) and CD43-APC (ebioscience, 84-3C1) antibodies (1:50) on PBS/0.5% BSA. CD34/CD43 double positive cells were considered for calculating differentiation efficiency. Once a continuous efficiency above 85% was obtained for at least two weeks, cultured human derived-microglial precursors were used for injections into post-implantation embryos for up to three

months. For embryo injection, cells were harvested from the supernatant and treated for 2 minutes with trypLE (Invitrogen) at 37ºC, washed with PBS and resuspended on media consisting of X-VIVO 15 with 10% FBS. Cell were kept on ice until injected. Immediately before injection, a 60µl drop of cell suspension was placed on a petri dish, cells were harvested by gentle suction under a stereoscope using a mouth pipette and injected into the amniotic cavity of E7.5 mouse embryos on pre-heated dissection media, taking care of introducing the microinjection needle through the exocoelomic cavity to avoid perforating the epiblast, 50-60 cells were injected per embryo. Finally, injected mouse embryos were grown in roller culture settings for 1-4 days according to the protocol described above for E7.5 embryos. Total number of integrated cells was measured using the cell counter Plugin in Fiji/ImageJ. The use of human ESC line follows the approval of Weizmann institute IRB-ESCRO (#1138-1, #856-1).

Bulk RNA-seq library preparation

V6.5 mESCs grown under naïve (ESCs) and primed (EpiSCS) conditions were used for RNA-seq analysis. Total RNA was extracted using the TRIzol-based RNA MiniPrep kit (Zymo Research). mRNA was purified using Poly-A Dynabeads mRNA DIRECT Kit (Invitrogen) and mRNA then was utilized for RNA-Seq by TruSeq RNA Sample Preparation Kit v2 (Illumina) according to manufacturer's instruction.

Bulk RNA-seq analysis

Bulk RNA-seq was measured from the following samples: mEpiSCs (2 biological replicates) and mESC (2 biological replicates). Reads were trimmed with TrimGalore 0.6.5 (flags --stringency 3 --paired) and aligned to GRCm38 genome using STAR aligner (flags --runThreadN 64 --genomeLoad). Counts were estimated using HTSeq-count 0.7.2 (flags -q -f bam -r pos -s no -t exon -i gene_name). Normalization and differentially expressed genes were calculated using DESeq2 R package, with default parameters. Differentially expressed genes were selected if their adjusted *p*-value was smaller than 0.01 and their Fold change was greater than 2. External gene signatures, based on mouse microarray data, were calculated from GSE60603 (PMID **25945737**)[7]: shortly, processed data were used to calculate t-test. Genes that had t-test *p*-value<0.05 were included in the gene signature. The overlap between differentially expressed gene signatures was calculated using Fisher exact test.

Supplementary Discussion

While E8.5 embryos were obtained from E6.5 mouse embryos cultured in previously reported static conditions[8] (50% DMEM/50% RS in 5% O_2), they could not be developed further toward E9.5 upon transfer to roller culture *ex utero* platform established herein. The latter might be a result of minor, yet notable, morphological differences between *in vitro* and *in vivo* embryos obtained (**Extended Data Fig. 6o**). Static culture does not sustain development of embryos beyond the early-somite stage (**Extended Data Fig. 6f**). Increasing gas pressure in static conditions (2.5 and 5 psi), addition of 5% Matrigel or supplementation with N2/B27 had a negative effect on embryo survival (**Extended Data Fig. 6i,j,m,n**). We also noted that culturing embryos under hypoxic conditions drastically decreased efficiency and embryo quality after two days compared to 21% O_2 (**Extended Data Fig. 6g**).

It is likely that the conditions described herein can be further improved to increase efficiency and quality in the future. We also cannot exclude that other different platforms, pressures or conditions might be devised in the future to enable enhanced outcome to the results shown herein. Future development of serum-free conditions might also be beneficial and help reduce variability.

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