Derivation and Maintenance of Mouse Expanded Potential Stem Cells

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Method Article

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Abstract

The zygote and blastomeres of cleavage stage mouse embryos have the capacity to differentiate to both embryonic and extra-embryonic lineages and are considered functionally totipotent. Until now, it has not been possible to establish stable cell lines that resemble these totipotent-like cells. Recently, we demonstrated that by modulating signalling pathways known to be important in early embryonic development it is possible to capture in vitro a self-renewing state that possessed features of preimplantation blastomeres. We reported that expanded potential stem cells \(EPSCs) can be established from 8-cell \(8C) embryos, individual 8-cell blastomeres, by direct conversion of mouse embryonic stem cells \(ESCs) and reprogrammed induced pluripotent stem cells \(iPSCs). Herein, we present a detailed protocol for the derivation and expansion of EPSCs in our novel media formulation, expanded potential stem cell medium \(EPSCM). It is envisioned that EPSCM and EPSCs will provide a useful resource to study the earliest stages of embryonic development and lineage commitment.

Introduction

By the time of implantation, three distinct cell lineages will have formed in the developing embryo. On the outside, enclosing the embryo lie the trophectoderm which is divided into mural and polar components. The mural trophectoderm surrounds the blastocoel of blastocyst and will have a key role in implantation and formation of primary giant cells. The polar trophectoderm located adjacent to the inner cell mass which give rise to the extra-embryonic ectoderm and ectoplacental cone. The inner cell mass consists of the epiblast and primitive endoderm with the epiblast located adjacent to the polar trophectoderm giving rise to all somatic cell types in the developing organism including the germ cell lineage. The primitive endoderm located adjacent to the blastocoel will form part of the yolk sac \(Yamanaka et al., 2006). From each of these three anatomical sites we can derive stem cells which retain the expression profile and growth factor requirements characteristic of the lineage from which it was derived \(Evans and Kaufman, 1981; Kunath et al., 2005; Martin, 1981; Tanaka et al., 1998). These stem cells have provided a biological resource to study the factors that induce and maintain their respective cellular identities. However, it remained unclear whether it was possible to experimentally derive and maintain stable cell lines from early embryos that contain features of cleavage-stage blastomeres. Recently, a transient subpopulation in embryonic stem cell \(ESC) culture that is thought to correspond to totipotent 2-cell \(2C)-stage blastomeres was reported \(Macfarlan et al., 2012). These 2C-like ESCs do not express pluripotency factors such as Pou5f1 \(Oct4), Sox2 or Nanog protein and demonstrate the ability to contribute to both embryonic and extra-embryonic tissues. The authors argue that entry into this ‘privileged state’ may partially be controlled by histone-modifying enzymes implicating epigenetics as a potential determinant of totipotency. Similarly, another group reported a rare Hhex positive subpopulation in ESCs cultured in 2i/LIF, a defined medium that maintains ESCs at an intrinsically stable ground state \(Wray et al., 2010; Ying et al., 2008) which could also apparently contribute to both trophoblast and embryonic lineage development \(Morgani et al., 2013). The aforementioned papers led one to the following conclusions. Firstly, cells with expanded potential exist in ESC culture conditions albeit transiently and at extremely low

percentages of the total cell population. Secondly, this infrequency may be explained by the fact that the culture condition is not optimized for the capture and expansion of these rare cell types. As stem cell populations can be established from the epiblast, primitive endoderm and polar trophectoderm, it may be reasonable to speculate; that if signalling pathways that are known to be active and critical for pre-implantation development could be selectively targeted using small molecule inhibitors, one may be able to enrich for or capture these cells with expanded potential from standard ESC cultures in the first instance. This hypothesis allowed us to formulate a novel media formulation called EPSCM which permitted the derivation of EPSCs from 8-cell stage preimplantation mouse embryos, single 8-cell stage blastomeres and by conversion of mouse ESCs and iPSCs. Expanded potential was defined based on the potential of EPSCs to contribute to the embryo proper, extraembryonic endoderm and placenta trophoblasts in chimeras. EPSCM maintained a homogenous population as a single EPSC could contribute to the aforementioned lineages in vivo. Furthermore, Bona fide trophoblast stem cell \(TSC) lines and extraembryonic endoderm stem \(XEN) cells could be directly derived from EPSCs in vitro. Detailed molecular analyses of the epigenome and single cell transcriptome revealed that EPSCs were enriched for a blastomere-specific transcriptomic signature and displayed a dynamic DNA methylome. The protocol described herein gives a comprehensive account of the steps involved in the derivation and expansion of mouse EPSCs.

Reagents

Media conditions o M10 • 500 ml Knockout –DMEM \(Invitrogen, 10829-018) • 50 ml Fetal Bovine Serum \(Hyclone) • 5.5 ml 100X MEM Non-essential amino acid \(Invitrogen, 11140) • 5.5 ml 100X Glutamine-Penicillin-Streptomycin \(Invitrogen, 10378) o M15 • 500 ml Knockout DMEM \(Invitrogen, 10829-018) • 90 ml Fetal Bovine Serum \(Hyclone) • 6 ml 100X MEM Non-essential amino acid \(Invitrogen, 11140) • 6 ml 100X Glutamine-Penicillin-Streptomycin \(Invitrogen, 10378) • 60 µl human LIF \(Millipore, LIF1010) • 4.3 µl b-mercaptoethanol \(Sigma, M6250) o N2B27-2i/LIF • NDiff 227 \(Stem Cells, SCS-SF-NB-02) • 1 µM PD0325901 \(Tocris, Cat. No. 4192) • 3 µM CHIR99021 \(Tocris, Cat. No. 4423) • 1 x 103 U/mL LIF \(Merck Millipore, LIF1010) o EPSCM • 400 ml DMEM/F12 \(Invitrogen 21331046), • 100 ml Knockout Serum Replacement \(Invitrogen 10828028), • 5 ml 100X Glutamine-Penicillin-Streptomycin \(Invitrogen, 10378) • 5 ml 100X MEM Non-essential amino acid \(Invitrogen, 11140) • 5 µl b-mercaptoethanol \(Sigma, M6250) • 50 µl human LIF \(Merck Millipore, LIF1010) • 1 µM PD0325901 \(Tocris, Cat. No. 4192) • 3 µM CHIR99021 \(Tocris, Cat. No. 4423) • 4 µM JNK Inhibitor VIII \(Tocris, Cat. No. 3222) • 10 µM SB203580 \(Tocris, Cat. No. 1402) • 0.3 µM A-419259 \(Santa Cruz, Cat. No. sc-361094) • 5 µM XAV939 \(Sigma, Cat. No. X3004). • 30 ng/mL Recombinant human IGFII \(R&D, 292-G2) \(Optional) o Freezing media • 80% EPSCM • 10% Fetal Bovine Serum \(Hyclone) • 10% Hybri-Max DMSO \(Sigma, D2650) Additional Reagents o Reagents for production and isolation of mouse embryos • Pregnant Mare Serum Gonadotropin \(Intervet, PMSG-Intervet) • Human Chorionic Gonadotropin \(Intervet, Chorulon) • KSOM medium \(Merck Millipore, MR-107-D) • Bovine serum albumin \(Sigma, A3311) • M2 medium \(Sigma, M7167) • Cytochalasin B \(CB) \(Sigma, C6762) • Tyrode's Solution \(Sigma, T1788) o Mouse embryonic

fibroblast feeders • Mitomycin C-inactivated SNL feeder cells o Dulbecco's Phosphate-Buffered Saline \(Invitrogen, 14190) o Accutase \(Merck Millipore, SCR005) o 0.1% Gelatin Solution

Equipment

o Incubator at 37°C with 95% air and 5% CO2 o Laminar flow hood o 37°C water bath o Leica dissection microscope o Leica DIML inverted microscope o Olympus IX81 inverted fluorescence microscope o Hand blown glass blunt pipette \(inner diameter: 40 µm) o 24-well tissue culture plates o 6-well tissue culture plates o 96-well tissue culture flat-bottomed plates.

Procedure

Preparation of SNL feeder plates Day 0: 1. Gelatinise plates by adding 1 ml, 0.5 ml and 100 µl per well of 0.1% gelatin to a 6-well, 24-well and 96-well plate respectively and leave the plates at room temperature for 1 hour. 2. Thaw frozen mitomycin-C treated SNL feeder cells from liquid nitrogen in a 37°C water bath. 3. Centrifuge the cryotube for 3 minutes @ 1200 rpm. 4. Aspirate gelatin from new plates. 5. Add M10 media to the new plates. a. 3 ml/well for 6-well plate. b. 1 ml/well for 24-well plate. c. 200 µl/well for 96-well plate 6. Carefully remove the supernatant and re-suspend cell pellet in fresh M10. 7. Equally distribute cell suspension between new plates and shake to ensure even distribution. The plating density of feeder cells should be approximately 8.4 x 104 / cm2. 8. Place feeder plates in incubator at 37°C with 95% air and 5% CO2. Day 1: 9. Feeder plates are ready for use. 10. Aspirate M10 and add EPSCM to feeder plates. a. 2 ml/well for 6-well plate. b. 500 µl /well for 24-well plate. c. 200 µl/well for 96-well plate. 11. Allow plates to equilibrate in incubator prior to cell plating. Embryo preparation 1. For the production of 4-cell or 8-cell embryos, CD1 or C57B6 females older than 8 weeks of age are superovulated by intraperitoneal injection of Pregnant Mare Serum Gonadotropin 7.5 IU each. 2. This is followed 46-48 hours later by injection of human Chorionic Gonadotropin 7.5 IU each, and these are then mated to proven studs. 3. Vaginal plugs are checked the following morning \(0.5 days post coitum, dpc). 4. 4-cell to 8-cell embryos are collected at 2.5 dpc by flushing the oviduct and the uterine cavity, respectively. 5. Embryos are cultured in 1.0 ml KSOM medium containing 3.0 □g/ml bovine serum albumin in an incubator with 5% CO2 at 37°C. Derivation of EPSC lines from whole 8C-stage embryos 6. Each 8-cell embryo is transferred to one well of a 96-well SNL feeder plate or a gelatinized plate in EPSCM. 7. Embryos are cultured in EPSCM for over 8 days until embryos hatch and form outgrowths. Until the embryos have attached media change is performed by the addition of fresh EPSCM to each well every 48-72 hours. Once outgrowth has attached, the media can be aspirated with the addition of fresh EPSCM. 8. To dissociate the outgrowth aspirate media from the wells and wash once with DPBS. Add Accutase 50 µl / well for a 96 well plate and place plate in incubator for 5 minutes. To assist in the dissociation gently pipette up anddown and across the plate. 9. Following confirmation of dissociation under the microscope, add 50 µl EPSCM \(equal volume to that of Accutase) to each well. 10. Transfer the 100 µl cell suspension \(50 µl Accutase + 50µl EPSCM) to a new pre-prepared 96-well SNL feeder plate or a gelatinized plate containing pre-equilibrated EPSCM. 11. After 2-3 days, EPSC colonies will reach 80-90%

confluence and are subsequently passaged into a 24 well SNL feeder plate or gelatinized plate and then expanded into 6 well plates. 12. For passaging from 24 well and 6 well plates, aspirate the media and wash once with DPBS. 13. Add Accutase 200 µl /well for a 24-well plate or 500 µl /well for a 6-well plate. 14. As before, place the plate in the incubator for 5 minutes and subsequently assist the dissociation with gentle pipetting. Following addition of an equal volume of EPSCM, transfer the cell suspension to a 15-ml falcon tube and centrifuge for 3 minutes at 1200 rpm. 15. Aspirate the supernatant and re-suspend in EPSCM. 16. Plate cells \(if required, counting can be performed at this point). Anticipated Results - Using SNL feeder layer, the EPSC derivation efficiency is close to 100%, whereas only about 20% will be achieved if embryos are plated in a gelatinized plate. See Figure 1 and Figure 2 for a representative temporal progression of 8-cell embryos in EPSCM. Derivation of EPSC lines from single 8C-stage blastomeres 1. Before separating the blastomeres, the 4-cell to 8-cell embryos are cultured in 1.0 mL KSOM containing 3.0 mg/mL BSA and 7.5 µg CB for at least half an hour. 2. The embryos are then suspended in 100 µl Tyrode's Solution to wash away KSOM media. 3. The embryos are then transferred into and suspended in a new 100 µl drop of Tyrode's Solution. 4. Immediately after the zona pellucida has dissolved in Tyrode's Solution, the embryos are transferred into a 100 µl M2 drop. 5. The embryos are washed a few times in M2 drops. 6. The embryos are then sucked and blown a few times by a blunt needle \(inner diameter: 40 µm). 7. The individual blastomeres are now collected. 8. For derivation of EPSC line from a single blastomere, individual blastomeres are plated into a 96-well plate on SNL feeders \(one blastomere per well). 9. The progress of blastomeres is monitored daily. As with preimplantation embryo culture, until the blastomere has attached, add fresh media to the well every 48-72 hours. Once attached, media can be carefully aspirated with the addition of fresh EPSCM. 10. At around day 7, some small cell clusters form and the cells continue to proliferate to form EPSC colony-like outgrowths. 11. By day 12, colonies are dissociated with Accutase and transferred into a new 96 well SNL feeder plate. 12. The cells are further expanded in 24-well and 6-well SNL feeder plates. Anticipated Results: The derivation efficiency on SNL feeders is approximately 28.1% \(9/32). See Figure 1a in the associated publication for a representative temporal progression of a single blastomere in EPSCM. Conversion of mouse ESCs / iPSCs to EPSCs Day 0 1. mESCs / miPSCs are plated on mitomycin C-inactivated SNL feeder cells in their original medium \(N2B27-2i/LIF or M15) at a low plating density \(1 x 103 cells / cm2). Day 1 2. Medium is switched to EPSCM and is changed daily. Day 3/4 3. Cells are passaged every 3–4 days at a 1:3 ratio. 4. As described above this is by a brief DPBS wash followed by single-cell dissociation using Accutase. Anticipated Results: After 5 passages, which takes approximately 15 days, the cells will functionally have acquired expanded potential. Note #1: In the associated publication, to detect the presence of EPSCs and conventional ESCs in chimeras, cells were made to express a CAG-mCherry, EF-1α-H2BmCherry or CAG-H2BmCherry expression cassette by PiggyBac transposon transposition using Amaxa nucleofection \(Lonza) according to the manufacturer's protocol for mouse ESCs \(mESC A-023). Note #2: Following plating of a single Rex1-GFP ESC on SNL feeders, the number of REX1-GFP positive colonies formed in EPSCM and in their original media \(M15 or 2i/LIF) were comparable indicating that EPSCM was permissive for mouse ESCs and did not select for a rare subpopulation of cells. See Figure 3 for representative examples of colony morphology on feeders and in feeder free conditions. Cryopreservation of EPSCs 1. Feed cells 2-3 hours before freezing. 2. Move all media to room temperature to warm up 20

minutes before starting. 3. Remove media from the plate and wash once with DPBS. 4. Remove DPBS and add Accutase to the plate. 5. Incubate the plate at 37°C for 5 minutes. 6. Tap the plate on the side to detach the cells from the plate. 7. Add fresh EPSCM of equal volume to that of Accutase. 8. Dissociate EPSCs by pipetting up and then down across the plate. 9. Transfer cell suspension to a new falcon tube. 10. Centrifuge the tube for 3 minutes @ 1200 rpm. 11. Carefully remove supernatant and re-suspend cell pellet in 500 µl freezing media. 12. Transfer 500 µl of cell suspension to each labelled cryotube. 13. Transfer tubes to freezing pot and store at -80°C for 24 hours. 14. After 24 hours transfer tubes to -180°C liquid nitrogen for long term storage. Cryorecovery of EPSCs Day -1: 1. Prepare SNL feeder plates. Day 0 2. Move media to room temperature to warm up 20 minutes before starting. 3. Transfer a vial of frozen cells to the 37°C water bath. 4. Once thawed, briefly centrifuge the tube to collect the pellet at the base of the cryotube. 5. Transfer the contents of the cryotube to a 50-ml falcon tube containing 15 ml of warmed EPSCM. 6. Centrifuge for 3 minutes @ 1200 rpm. 7. Carefully remove supernatant and re-suspend cell pellet in EPSCM. 8. Plate cells. Day 1 9. 24 hours after thawing, change media to EPSCM. 10. Change media every day until the cells are ready to be passaged.

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Figures

Figure 1

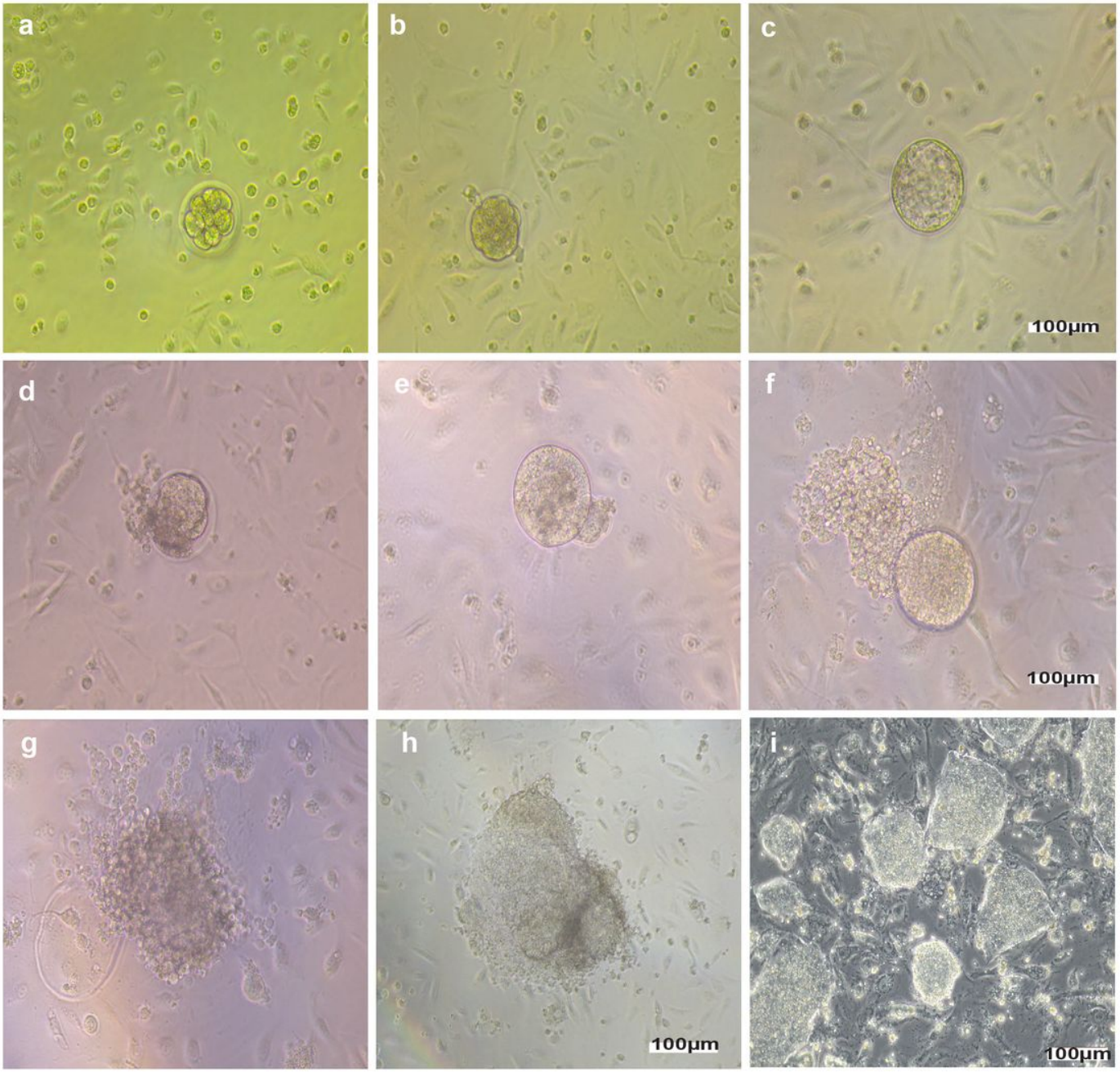

Figure 1

Derivation of EPSCs from 8-cell embryos on SNL feeders. Embryos were cultured on SNL feeders in EPSCM. Normal developmental progression was initially seen with formation of morula and blastocyst. a. 0 hour; b. 24 hours; c. 48 hours. By 96 hours the late blastocyst became filled with expanding cells. d. 96 hours; e. 120 hours hatching was observed and the cells attached to feeders forming a primary

outgrowth. f. 144 hours; g. 168 hours; h. day 13. i. EPSC lines were established by passaging the primary outgrowths in EPSCM.

Figure 2

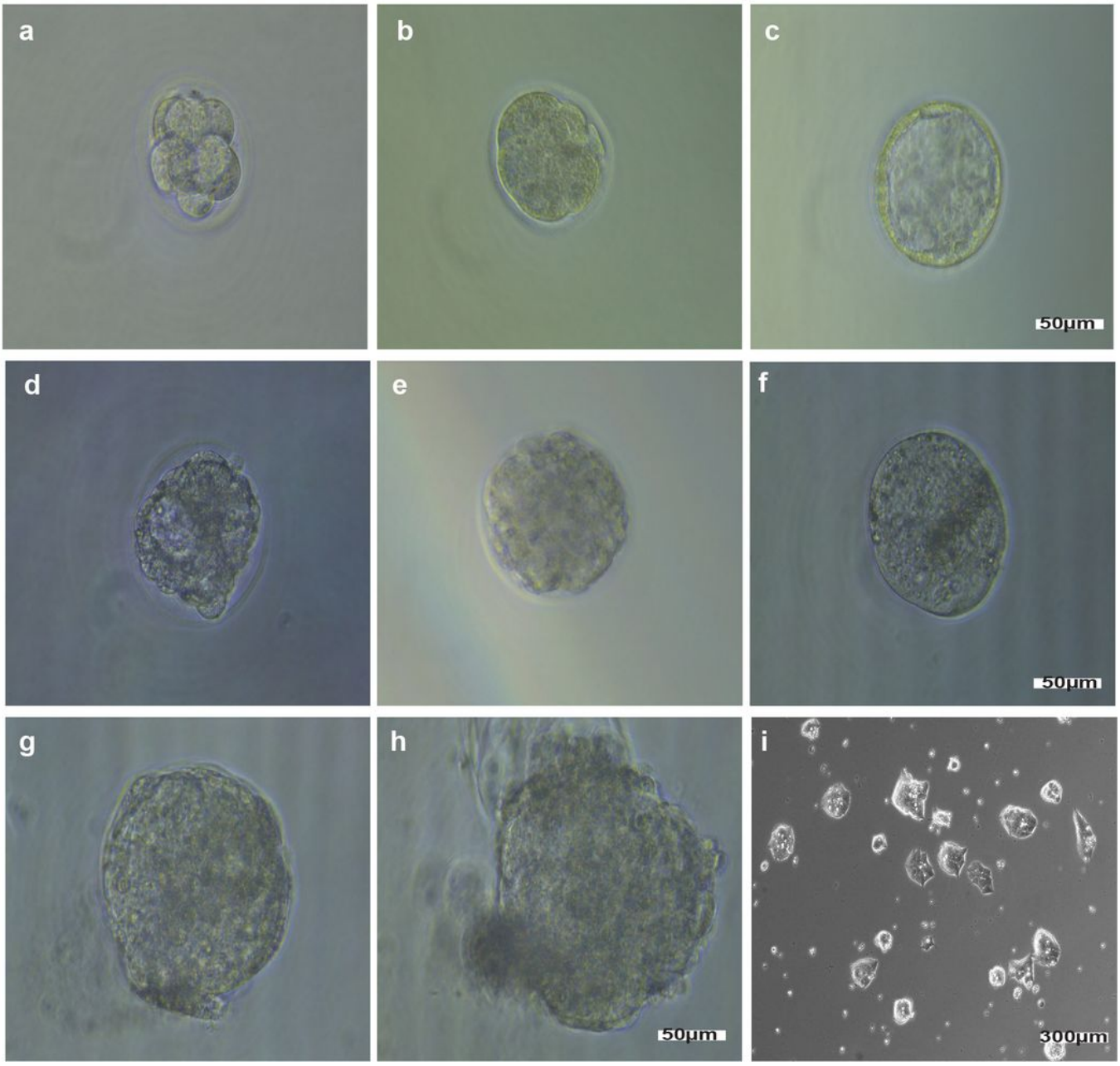

Figure 2

Derivation of feeder free EPSCs from 8-cell embryos. Embryos were cultured on 0.1% gelatin coated 96-well plates in EPSCM. Normal developmental progression was initially seen with formation of morula and blastocyst. a. 0 hour; b. 24 hours; c. 48 hours. At 72 hours, the blastocoele began to collapse, by 96 hours

the late blastocyst became filled with expanding cells. d. 72 hours; e. 96 hours; f. 168 hours hatching was observed and the cells attached to feeder forming a primary outgrowth. g. day 10; h. day 11. i. EPSC lines were established by passaging the primary outgrowths in EPSCM.

Figure 3

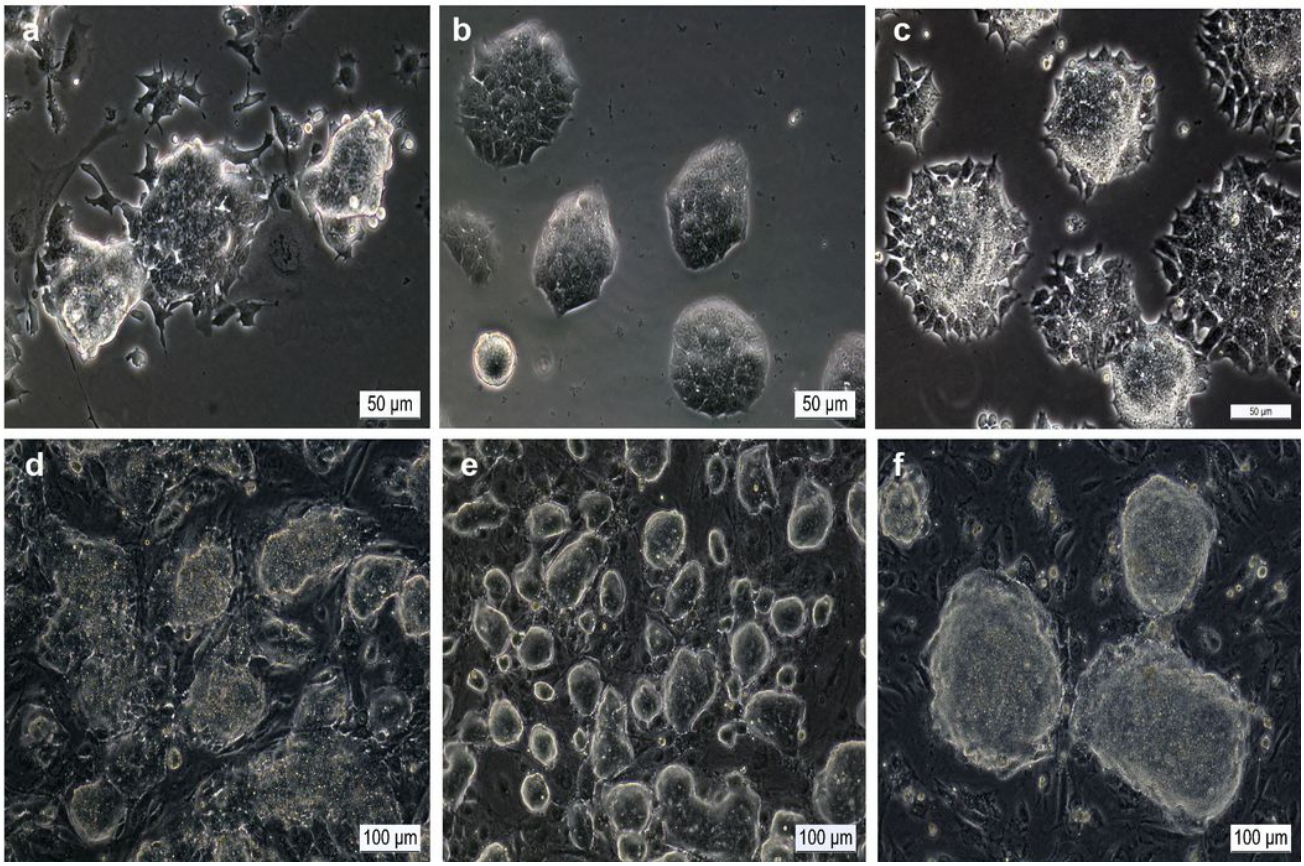

Figure 3

Conversion of ESCs to EPSCs. E14Tg2a mouse ESCs were cultured in EPSCM for 5 passages on 0.1% gelatin coated plates or on SNL feeders. a-c. Representative colony morphology of ESCs and EPSCs on gelatin coated plates. a. ESCs in M15; b. ESCs in 2i/LIF; c. EPSCs in EPSCM. d-f. Representative colony morphology of ESCs and EPSCs on SNL feeders. d. ESCs in M15; e. ESCs in 2i/LIF; f. EPSCs in EPSCM.