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Derivation of extraembryonic endoderm stem (XEN) cells from mouse embryos and embryonic stem cells

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Abstract

At the time of implantation in the maternal uterus, the mouse blastocyst possesses an inner cell mass comprising two lineages: epiblast (Epi) and primitive endoderm (PrE). Representative stem cells derived from these two cell lineages can be expanded and maintained indefinitely *in vitro* as either embryonic stem (ES) or XEN cells, respectively. Here we describe protocols that can be used to establish XEN cell lines. These include the establishment of XEN cells from blastocyst-stage embryos in either standard embryonic or trophoblast stem (TS) cell culture conditions. We also describe protocols for establishing XEN cells directly from ES cells by either retinoic acid and activin-based conversion or by overexpression of the GATA transcription factor Gata6. XEN cells are a useful model of PrE cells, with which they share gene expression, differentiation potential and lineage restriction. The robust protocols for deriving XEN cells described here can be completed within 2–3 weeks.

Introduction

The mouse embryo ~3.5 d after fertilization forms a blastocyst comprising three lineages[1]: the extraembryonic trophectoderm (TE), the PrE and the pluripotent Epi (Fig. 1) from which cognate *ex vivo* stem cells can be derived. TS cells are derived from the TE[2], XEN cells from the PrE[3] and ES cells from the Epi (refs. 4,5; Fig. 2) (reviewed in ref. 6). Notably, each of these stem cell lines is a useful model of the blastocyst cell lineage that they represent. Mouse ES and TS cells have been used successfully for many years to model Epi or TE biology, including the mechanisms of pluripotency maintenance and placental development, respectively. Recently derived XEN cell lines have the distinctive characteristic of cells with at least two morphologies: they are highly refractile as well as epitheliallike[3] (Fig. 2), and

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they are only beginning to be used to understand the mechanisms of PrE development with significance for stem cell and developmental biology.

Mouse ES cells can be directed to differentiate into extraembryonic lineages by the overexpression of single transcription factors, such as the caudal-related homeodomain transcription factor Cdx2 (to derive TS cells)[7] or the GATA transcription factor Gata6 (to derive XEN cells)[8]. Retinoic acid treatment of mouse ES cells[9–11] or embryoid body aggregation[12] has been shown to promote a heterogeneous mixture of XEN-differentiated cells. Notably, these cells have not been demonstrated to self-renew indefinitely, unlike bona fide XEN cell lines. We have recently demonstrated that mouse ES cells can be converted to stable XEN cell lines using retinoic acid together with activin[13]. In this protocol, we focus on the derivation of XEN cells from embryos and ES cells.

The molecular mechanisms underlying XEN cell establishment and maintenance are beginning to be understood. Robust methods for XEN cell derivation from embryos and ES cells, as well as the concomitant availability of XEN cell lines, will further facilitate and improve our understanding of the key fate decisions that occur within the early embryo, including unraveling mechanisms underlying cellular differentiation and pluripotency[14,15]. As a stem cell type that can be derived from both embryos and ES cells, XEN cells are emerging as a valuable tool for modeling the XEN lineage.

Applications of XEN cells

XEN cell derivation can be used as a phenotypic tool to assess the requirement of genes for XEN cell specification, maintenance or expansion, as we have previously demonstrated for SRY-box containing gene 17 *Sox17*)[4], platelet-derived growth factor receptor alpha (*Pdgfra*)[11] and fibroblast growth factor 4 (Fgf4)[16]. Notably, established XEN cell lines serve as a paradigm for XEN biology and for the differentiation of the PrE into derivatives such as visceral[17–19] and parietal endoderm.

Given the fact that reciprocal inductive interactions between the pluripotent Epi and its adjacent extraembryonic tissues, including the visceral endoderm (reviewed in refs. 20 and 21), are crucial to patterning the early embryo, XEN cells can be used as an *ex vivo* tool for teasing apart the underlying mechanisms and for identifying the key molecules involved[12]. XEN cells can be used as an important *in vitro* tool for elucidating details of additional patterning activities of the extraembryonic endoderm, such as identifying factors involved in cardiac induction[22–24]. Moreover, as they can be propagated in large quantities and do not require growth factor supplements to culture media, these cells are a cost-effective, attractive and tractable system for high-throughput analyses. They can be used in screens for PrE-differentiating factors or in proteomics analyses to identify secreted factors that mediate tissue patterning (for example, during cardiac induction)[22–24]. XEN cells show paternally imprinted X-chromosome inactivation[3], and thus they serve as a useful model for understanding the molecular basis of post-translational and epigentic modifications[25]. Furthermore, as the extraembryonic endoderm has recently been shown to contribute to the gut endoderm *in vivo*[26], XEN cells may also serve as an alternative self-renewing source of definitive endoderm-derived cells and tissues for regenerative medicine. Intriguingly, XEN cells rapidly silence retroviral transcription compared with mouse ES and TS cells and may therefore also represent a useful model to study how viral gene expression is regulated[27].

XEN cell establishment from embryos

Several protocols describe the derivation of XEN cells from mouse embryos, including the direct plating of blastocyst stage embryos[3] or immunosurgery, to isolate and to plate the inner cell mass[28,29]. In 2005, Kunath *et al.*[3] reported the first isolation and characterization

of XEN cells using methods based on existing protocols of deriving ES[28] and TS cells[2,30,31]. In this case, embryonic day 3.5 blastocysts were cultured on mouse embryonic fibroblast (MEF) cells until they formed an outgrowth that, as in routinely used ES and TS cell derivation protocols, was subsequently disaggregated to promote the proliferation of the different cell types present in the blastocyst. In both procedures, XEN cell outgrowths could be observed alongside ES or TS cells, respectively, suggesting that these conditions are also permissive for XEN cell derivation.

Our experience has been that slight modifications to these protocols (especially media conditions and time of disaggregation) can favor the propagation of XEN cells over that of ES or TS cells. We have also found that, in our hands, TS cell conditions are generally less efficient for the derivation of XEN cell lines compared with ES cell conditions (21% and 56% efficiency, respectively). ES cells start appearing from the outgrowths first and are less resilient, which might facilitate the ability of XEN cells to outcompete them in a culture dish. TS cells, in contrast, reach their proliferative peak around the same time as XEN cells and tend to outcompete XEN cells present in the same cultures, especially in the presence of recombinant FGF4 (ref. 2) or FGF2 (bFGF), the latter of which can elicit the same effect[31] and is slightly more cost effective.

XEN cell establishment by transcription factor overexpression

In classical experiments, Davis *et al.*[32] demonstrated that the expression of a single transcription factor, MyoD, was sufficient to convert fibroblasts into myogenic cells. More recently, transcription factor– mediated cell-fate switches have been demonstrated for several other cell types, including the reprogramming of fibroblasts into induced pluripotent stem cells via ectopic expression of the POU domain, class 5, transcription factor 1 (Pou5f1, also known as Oct4), Klf4, Sox2 and Myc (also known as c-Myc)[33].

Ectopic transcription factor expression has also been shown to induce the conversion of XEN cells from ES cells (reviewed in ref. 6). Niwa and colleagues[8,34] demonstrated that the expression of Gata4 or Gata6 alone is sufficient to induce the conversion of ES cells into XEN cells. Notably, these GATA-derived XEN cells share the molecular and functional characteristics of embryo-derived XEN cells, including contribution to PrE lineages in chimeric embryos[34]. XEN-like cells have also been generated by ectopic expression of Sox17 (refs. 14,35–37). It is unclear whether Sox17 alone is sufficient to drive XEN cell commitment, as these cells retain the expression of ES cell–associated genes, such as *Nanog* and *Oct4* (refs. 14,35,38), and their contribution to chimeras has not yet been reported. Moreover, it is unclear whether alternative PrE-associated transcription factors can be used to convert ES to XEN cells.

The simplest method used to derive XEN cells from ES cells is the expression of *Gata6* either by transfection of a circular plasmid or a linearized DNA using chemical or nonchemical (e.g., electroporation) methods. There are several chemical-based transfection approaches. The most widely used methods for ES cells are lipidbased lipopolyamines[39] and cationic polymer–based transfection (reviewed in ref. 40). Lipopolyamines (e.g., lipofection) work by coating a *Gata6*-cDNA plasmid with a cationic lipid, allowing the DNA to cross the negatively charged phospholipid ES cell membrane by endocytosis[41,42]. Cationic polymers (e.g., Xfect) act by binding DNA and condensing it, thereby facilitating DNA entry into the cell cytoplasm[40,43]. An advantage of lipopolyamines and cationic polymers is that they do not require specialized equipment, although the reagents may be cytotoxic and the ratio of DNA to transfection reagent needs to be optimized to the specific cell type[42] (reviewed in ref. 44).

Electroporation is the principal alternative to chemical-based transfection approaches and is routinely used for ES cell transfection[45–47]. Electroporation allows for permeabilization of the ES cell membrane and transfection of a *Gata6* cDNA via the application of a transient electrical field. Depending on the purity of the DNA preparation, this can be a highly efficient method and is most commonly used for gene targeting of ES cells. The choice of whether the *Gata6* cDNA plasmid is introduced as a circular plasmid or as a linearized DNA can affect transfection efficiency, as supercoiled or open-circular DNA is optimal for transient expression, whereas linearized DNA is more recombinogenic and best suited for stable transfection[48–50].

Continued passage of transiently transfected cells will result in the dilution of the exogenous DNA and may restrict the time window for subsequent analysis. Therefore, although it is initially more time consuming, the generation of stable transfected ES cells that have integrated a linearized *Gata6* cDNA into the genome can be experimentally more consistent compared with transient transfection. By including a drug-resistance gene in the *Gata6* cDNA–targeting plasmid, drug selection can be used to expand only targeted ES cells that retain the ability to overexpress the *Gata6* cDNA. Another important consideration is the use of an efficient and robust promoter to drive exogenous *Gata6* expression. In mouse ES cells, the cytomegalovirus (*CMV*) promoter may be silenced[51]. In contrast, the ubiquitin C (*UBC*) promoter, elongation factor 1α(*EF1A*) promoter, and the chicken β-actin (*ACTB*) promoter coupled with the CMV early enhancer (*CAGG*) have been reported to be more robustly expressed[51].

Although these are not described here, alternative approaches for *Gata6* overexpression include viral transduction. Owing to potential toxicity, viral production and transduction require at least a category II tissue culture facility, and the silencing of retroviral transcription in XEN cells may obviate this approach[27]. Moreover, reversible overexpression of *Gata6* can be engineered using an inducible expression system containing a tetracycline-responsive element (controlled by tetracycline or doxycycline), a mutant estrogen-responsive element (controlled by 4-hydroxytamoxifen) or a glucocorticoid-responsive element (controlled by dexamethasone). When combined with drug selection, an inducible system is an effective means of overexpressing *Gata6* simultaneously in all cells. Although overexpression of *Gata6* may initially be a simpler method to drive XEN differentiation, a major note of caution is that its ubiquitous expression may hinder the ability of converted XEN cells to differentiate into subtypes of XEN cells. One major limitation may be that constitutive *Gata6* expression blocks differentiation of induced XEN cells into visceral endoderm subtypes, as *in vivo* these cells lack *Gata6* expression while retaining the expression of *Gata4* (refs. 52,53). Here we describe a method to constitutively express *Gata6* in mouse ES cells, which can be modified to overexpress other genes such as *Gata4* or to introduce an inducible expression system as has been described previously[34,54,55]. Moreover, transposon systems such as *piggyBac*[56] may also be used, although these methods have not yet been shown to convert ES to XEN cells.

XEN cell establishment by growth factor conversion of ES cells

Although ES cells largely contribute to the Epi in chimera embryos, it has been observed that committed XEN and TS cells also arise within pluripotent culture conditions[14,57,58]. The presence of TS-and XEN-like cells within ES cell cultures suggests that ES cells may have a broader cell fate potential and are therefore able to differentiate into stable extraembryonic stem cell lines directly. Indeed, ES cell aggregation results in the formation of embryoid bodies with an outer layer of XEN cells[12], and growth factors such as retinoic acid have been shown to differentiate embryonic carcinoma and ES cells into XEN-like cells[9–11,59]. Moreover, the culture of ES cells in serumfree medium on fibronectin-coated

dishes at high density has been suggested to promote visceral endoderm differentiation[60]. However, it was unclear whether self-renewing XEN cells could be differentiated directly from mouse ES cells without gene manipulation. We have recently expanded on these studies by developing a technique to convert ES cells directly into stable XEN (cXEN) cell lines that are equivalent to embryo-derived XEN cells[13]. We previously demonstrated that cXEN cells are molecularly indistinguishable from embryo-derived XEN and iXEN (i.e., transcription factor induced) cells and are equivalently responsive to differentiation-promoting factors (i.e., bone morphogenetic protein (BMP)-induced visceral endoderm differentiation). Our efforts to generate chimera embryos from cXEN cells resulted in contribution to the parietal endoderm at early postimplantation stages (K.K.N., M. Kang and A.-K.H., unpublished observations), as has been reported for XEN cells derived from embryos[3]. Here we describe our method for converting mouse ES cells into stable cXEN cell lines using growth factors. Specifically, we use retinoic acid together with activin, which has been demonstrated to promote primitive endoderm development *in vivo* and *in vitro*[17,61]. Notably, although retinoic acid and activin have been shown to differentiate ES cells into neurons and definitive endoderm, respectively, we find that the combination of both factors promotes XEN cell differentiation. We previously tested a range of retinoic acid concentrations from 0 to 100 μM and a range of activin concentrations from 0 to 20 ng ml^{-1}. Although XEN-like cells can emerge in the absence of exogenous retinoic acid and activin[14], the proportion of XEN-like cells is enhanced (40.3%) in 0.01 μM retinoic acid and 10 ng ml^{-1} activin[13]. We therefore use the lowest dose of retinoic acid and activin that gives us the highest efficacy of XEN cell emergence, although it is presently unclear whether different concentrations of these growth factors influence the identity of the XEN cells obtained. Thus far, we have been able to derive XEN cell lines from any mouse ES cell line and mouse strain unless the cells are mutant for a gene required for XEN establishment and/or maintenance[13], although the speed of conversion can vary with any given cell line. An important consideration is the initial heterogeneity that emerges after growth factor treatment. As with the protocol for XEN cell derivation from embryos, selecting cells with XEN cell morphology facilitates the expansion of stable cXEN cells that are able to self-renew indefinitely. This ES cell conversion protocol is particularly useful for determining the genetic requirement for XEN cell function using mutant mouse ES cells without the necessity for mutant mice.

MATERIALS

REAGENTS

Derivation from embryos using modified ES cell conditions—

- Pregnant mouse, 3.5 days post coitum (d.p.c.) **! CAUTION** Experiments involving rodents must conform to all relevant institutional and governmental regulations.
- Mitotically inactivated MEF cells (Reagent Setup)
- MEF medium (DMEM supplemented with FBS (Gibco, cat. no. 10082147), L-glutamine, β-mercaptoethanol, MEM non-essential amino acids and sodium pyruvate. Optionally, add penicillin-streptomycin; see Reagent Setup)
- M2 medium (Millipore, cat. no. MR-015-D)
- ES cell medium (DMEM supplemented with FBS (ES cell qualified; Gibco, cat. no. 16141079), L-glutamine (Gibco, cat. no. 25030-081), β-mercaptoethanol (Sigma-Aldrich, cat. no. M6250), MEM non-essential amino acids (Gibco, cat. no. 11140035), sodium pyruvate (Gibco, cat. no. 11360-070), leukemia inhibitory factor (LIF; Millipore, cat. no. ESG1106); optional: penicillin- streptomycin (Invitrogen, cat. no. 15140122); see Reagent Setup)

- Ethanol, 70% (vol/vol; Sigma-Aldrich, cat. no. E7023) **! CAUTION** Ethanol is flammable and may be harmful if inhaled or ingested.
- PBS, 1×, sterile (Ca^{2+} and Mg^{2+} free; Gibco, cat. no. 14190-094)
- Trypsin-EDTA, 0.25% (wt/vol; Gibco, cat. no. 25200-056)

Derivation from embryos using modified TS cell conditions—

- Pregnant mouse, 3.5 d.p.c.
- Mitotically inactivated MEF cells (Reagent Setup)
- TS cell medium (includes advanced RPMI 1640 (Gibco, 12633-012), FBS (Gibco, cat. no. 10082147), L-glutamine, β-mercaptoethanol, sodium pyruvate; optional: 1% (vol/vol) penicillin-streptomycin. Aliquots are supplemented with recombinant FGF2 or FGF4 (Sigma-Aldrich, cat. no. F8424) and heparin (Sigma-Aldrich, cat. no. H3393); see Reagent Setup)
- MEF-conditioned medium (Reagent Setup)
- M2 medium (Millipore, cat. no. MR-015-D)
- Ethanol, 70% (vol/vol; Sigma-Aldrich, cat. no. E7023) **! CAUTION** Ethanol is flammable and may be harmful if inhaled or ingested.
- PBS, 1×, sterile (Ca^{2+} and Mg^{2+} free; Gibco, cat. no. 14190-094)
- Trypsin-EDTA, 0.25% (wt/vol; Gibco, cat. no. 25200-056)

Derivation from mouse ES cells by *Gata6* overexpression—

- Mitotically inactivated MEF cells (Reagent Setup)
- MEF medium (Reagent Setup)
- ES cells (Reagent Setup)
- ES cell medium (Reagent Setup)
- Ethanol, 70% (vol/vol; Sigma-Aldrich, cat. no. E7023) **! CAUTION** Ethanol is flammable and may be harmful if inhaled or ingested.
- Gelatin, 0.1% (wt/vol; Sigma-Aldrich, cat. no. G1890) made up in sterile dH_2O (Sigma-Aldrich, cat. no. W1503) and autoclaved to dissolve
- PBS, 1×, sterile (Ca^{2+} and Mg^{2+} free; Gibco, cat. no. 14190-094)
- Trypsin-EDTA, 0.05% (wt/vol; Gibco, cat. no. 25300054)
- Xfect (Clontech, cat. no. 631320) or Lipofectamine 2000 (Invitrogen, cat. no. 11668030)
- Opti-MEM I (Invitrogen, cat. no. 51985)
- Bio-Rad Gene Pulser II Xcell eukaryotic system (Bio-Rad, cat. no. 377267)
- Cuvette, 4 mm (Cell Projects, cat. no. EP-104)
- Agarose (Sigma-Aldrich, cat. no. A9414)
- Maxi-prep kit (Qiagen, cat. no. 12262)
- *Gata6* cDNA vector (Origene, cat. no. MC219384)

- Isopropanol (Sigma-Aldrich, cat. no. I9516)
- Sodium acetate (Fluka, cat. no. 71196)
- Wizard SV genomic DNA purification system (Promega, cat. no. A2360)

Derivation from mouse ES cells using growth factors—

- cXEN derivation medium (standard XEN medium supplemented with all-*trans* retinoic acid (Sigma-Aldrich, cat. no. R2625) dissolved in DMSO plus activin A (R & D Systems, cat. no. 338-AC-010); Reagent Setup)
- Mitotically inactivated MEF cells (Reagent Setup)
- MEF medium (Reagent Setup)
- ES cells (Reagent Setup)
- ES cell medium (Reagent Setup)
- Ethanol, 70% (vol/vol; Sigma-Aldrich, cat. no. E7023) **! CAUTION** Ethanol is flammable and may be harmful if inhaled or ingested.
- Gelatin, 0.1% (wt/vol; Sigma-Aldrich, cat. no. G1890) made up in sterile dH_2O (Sigma-Aldrich, cat. no. W1503) and autoclaved to dissolve
- PBS, 1×, sterile (Ca^{2+} and Mg^{2+} free; Gibco, cat. no. 14190-094)
- Trypsin-EDTA, 0.05% (wt/vol; Gibco, cat. no. 25300054)

EQUIPMENT

- Conical tubes (15 ml; BD Falcon, cat. no. 352095)
- Conical tubes (50 ml; BD Falcon, cat. no. 352070)
- Tissue culture–treated plate (six well; Corning, cat. no. 3516)
- Tissue culture–treated plate (12 well; Corning, cat. no. 3512)
- Tissue culture–treated plate (24 well; Corning, cat. no. 3524)
- Tissue culture–treated dish (100 mm; Corning, cat. no. 430167)
- Tissue culture–treated dish (150 mm; Corning, cat. no. 430599)
- Tissue culture–treated plate (96-well flat bottom; Corning, cat. no. 3595)
- Tissue culture–treated plate (96-well round bottom; Corning, cat. no. 3799)
- Plate (four well; Nunc, cat. no. 12566300)
- Cryotube vials (Nunc, cat. no. 144444)
- Filter, 0.22 μm (Corning, cat. no. 431097)
- Flame-pulled glass Pasteur pipettes (unpulled; e.g., Fisher Scientific, 13-678-20D)
- Dissecting microscope (e.g., Leica M80)
- Inverted microscope (e.g., Olympus CKX41)
- Water bath (e.g., Grant JB Aqua 12)
- Centrifuge (e.g., Eppendorf Centrifuge 5804)
- Hemocytometer (e.g., Hausser scientific, cat. no. 3110)

- CO_2 incubator (e.g., Sanyo)

Dissection tools—

- Scissors (e.g., Roboz, cat. no. RS-5910)
- Forceps (e.g., Dumont no. 5, Roboz; cat. no. RS-4976)
- Syringes with 27-gauge needles (10 ml; BD Biosciences, cat. no. 309623)

Mouth-controlled pipette (Reagent Setup)—

- Plastic mouthpiece (MedTech, cat. no. 1501P-B4036-2)
- Rubber tubing (Fisher Scientific, cat. no. 22-362-772)
- Filtered pipette tip, P1000 (USA Scientific, cat. no. 1126-7810)

REAGENT SETUP

Mitotically inactivated MEF cells—Mitotically inactivated MEFs should be plated on pregelatinized cell culture–treated plates 1 d before initiating XEN derivation MEFs can be derived from CF1 mice that are dissected 12.5–13.5 d.p.c.; cells are passaged 3–5 times before inactivation, either by γirradiation or by mitomycin C treatment.

ES cells—ES cells should be thawed ~1–2 weeks before initiating XEN differentiation and passaged at least twice. ES cells are typically maintained on MEFs.

ES cell medium—Prepare a solution of the following: DMEM supplemented with 15% (vol/vol) FBS (ES cell qualified), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 0.1 mM MEM non-essential amino acids, 1 mM sodium pyruvate and 10^3 IU leukemia inhibitory factor. Optionally, add 1% (vol/vol) penicillin-streptomycin. Medium should be prepared in a sterile tissue culture hood and filter sterilized. The medium can be stored for up to 1 month at 4 °C.

MEF medium—Prepare a solution of the following: DMEM supplemented with 15% (vol/vol) FBS, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 0.1 mM MEM non-essential amino acids and 1 mM sodium pyruvate. Optionally, add 1% (vol/vol) penicillin-streptomycin. Medium should be prepared in a sterile tissue culture hood and filter sterilized. The medium can be stored for up to 1 month at 4 °C.

TS cell medium—Prepare a solution of the following: advanced RPMI 1640 supplemented with 20% (vol/vol) FBS, 2 mM L-glutamine , 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate; optional: 1% (vol/vol) penicillinstreptomycin. Medium should be prepared in a sterile tissue culture hood and filter sterilized. The medium can be stored for up to 1 month at 4 °C. Prepare a freshly made aliquot supplemented with 24 ng ml^{-1} recombinant FGF2 or FGF4 and 1 μg ml^{-1} heparin.

MEF-conditioned medium—Preparation of MEF-conditioned medium should begin at least 10 d before initiating the XEN derivation protocol B. Thaw and culture three 150-mm tissue culture plates of mitotically inactivated MEFs in 25 ml of standard TS cell medium (not supplemented with FGF2/FGF4 and heparin) for 3 d. Collect the medium in 50-ml conical tubes and store the medium at − 20 °C. Add another 25 ml of TS cell medium to each plate and repeat the procedure twice until 225 ml of MEF-conditioned medium have been collected. Thaw the frozen batches and centrifuge the medium at 2,300*g* for 20 min at

room temperature (20–25 °C) to remove debris. Filter-sterilize the medium and store it at 4 °C.

Standard XEN medium—Prepare a solution of advanced RPMI 1640 supplemented with 15% (vol/vol) FBS and 0.1 mM β-mercaptoethanol. Optionally, add 1% (vol/vol) penicillin-streptomycin. Medium should be prepared in a sterile tissue culture hood and filter sterilized. The medium can be stored for up to 1 month at 4 °C. Advanced RPMI 1640 contains glutamine; alternatively, RPMI medium can be used and requires the addition of 2 mM L-glutamine.

cXEN derivation medium—Freshly prepare a solution of standard XEN medium supplemented with 0.01 μM all-*trans* retinoic acid dissolved in DMSO plus 10 ng ml^{-1} activin A. A wide range of retinoic acid concentrations can be used, ranging from 0.01 to 10 μM. The derivation medium can also be supplemented with 24 ng ml^{-1} recombinant FGF2 or FGF4 and 1 μg ml^{-1} heparin, although this is not required if endogenous *Fgf4* is intact. **! CAUTION** Retinoic acid is light sensitive and may be harmful if ingested or absorbed.

Freezing medium—Freshly prepare a solution of 10% (vol/vol) DMSO (Sigma-Aldrich, cat. no. D2650) and 90% (vol/vol) FBS. Keep the solution on ice or at 4 °C until immediately before use.

***Gata6* cDNA for transfection—**Prepared as described in Box 1.

EQUIPMENT SETUP

Mouth-controlled pipette—Assemble a mouth-controlled pipette. This is used for handling embryos and for dissociating blastocyst *in vitro*–matured outgrowths. Mouth-controlled pipettes can be assembled in a variety of designs (for variations see Nagy *et al.*[28]). A simple mouth pipettor can be made by inserting the pointed end of a P1000 filter tip into one end of a cut rubber tube and attaching a plastic mouthpiece to the other. A pulled glass Pasteur pipette can now be attached to the filter tip. Pasteur pipettes can be pulled either over a flame or by using a micropipette puller (Fig. 3).

PROCEDURE

Preparation of MEF feeders ● TIMING 15–30 min

1| Pregelatinize tissue culture plates for >10 min with 1–2 ml of 0.1% (wt/vol) gelatin. Aspirate the gelatin before seeding the cells.

? TROUBLESHOOTING

2| One day before starting the derivation, place a frozen vial of mitotically inactivated MEFs in a 37 °C water bath until nearly thawed and transfer the contents into a 15-ml Falcon tube containing 5 ml of MEF medium. Centrifuge the medium at 200*g* for 4 min at room temperature and aspirate the supernatant.

? TROUBLESHOOTING

3| Resuspend the cell pellet in MEF medium and plate it at a density of 6×10^4 MEFs per well for a four-well plate, 1.3×10^5 MEFs per well for a six-well plate or 7.8×10^5 cells per 100-mm tissue culture plate, and then incubate the plates overnight at 37 °C. The number of plates to prepare depends on the number of XEN cell derivations to be performed. Generally, four plates (4-well plates) per pregnant mouse for Steps 6A and 6B are sufficient, or a six-well plate for Steps 6C–F.

? TROUBLESHOOTING

4| The morning before starting the derivation, aspirate the medium on the MEF feeders and replace it with ES cell medium for derivation using ES cell conditions (Step 6A), with TS cell medium for derivation using TS cell conditions (Step 6B) or with XEN cell medium for derivation from ES cells (Steps 6C–F). When required, plate MEFs that carry drug-resistance genes, such as DR4 MEFs (resistant to neomycin, hygromycin, puromycin and Zeocin) (Table 1).

? TROUBLESHOOTING

Cell preparation

5| If you are deriving cells using ES or TS cell conditions, follow option A to collect E3.5 blastocysts. If you are deriving XEN cells from ES cells, follow option B.

(A) Collecting E3.5 blastocysts (flushing)

i. Dissect out the uterine horns of a pregnant mouse at 3.5 d.p.c. and place them on the lid of a 100-mm culture dish in a drop of M2 medium.

ii. Under a dissection microscope (approximately ×10 total magnification), remove large fat pads and cut away the ovaries and oviducts. Place each of the cleaned uterine horns in a fresh drop of M2 medium.

iii. With a 1-ml syringe, take up 0.5 ml of M2 medium and attach a needle (27 gauge). While securing the uterine horn with forceps, insert the needle and flush the uterine horn with ~0.2 ml of M2 medium (Fig. 3) and remove the uterine horn from the drop. Repeat this process with the second uterine horn.

iv. Under higher magnification (~×30–×40), find the flushed blastocysts in both drops.

? TROUBLESHOOTING

v. By using a mouth-controlled pipette, transfer the blastocysts to a new drop of M2 medium. Repeat the step twice to wash away debris, lipid drops and blood cells.

vi. Proceed with the derivation of XEN cells using either option A (ES cell conditions) or option B (TS cell conditions) in Step 6.

(B) ES cell maintenance in medium with serum

i. Thaw the ES cells rapidly in a 37 °C water bath and transfer them into a 15-ml Falcon tube containing 5 ml of MEF medium.

ii. Centrifuge the tube at 200*g* for 4 min at room temperature, aspirate the supernatant and resuspend the ES cell pellet using 1 ml of ES cell medium.

iii. Aspirate the medium on the irradiated MEFs and replace it with ES cell medium. Plate ~3×10^5 ES cells onto MEFs in one well of a six-well plate or 2×10^6 ES cells on a 100-mm tissue culture dish. Incubate the cells at 37 °C.

▲ **CRITICAL STEP** All 37 °C incubations should be carried out in a 5% CO_2 humidified incubator.

iv. Change the ES cell medium daily to maintain the cell line and monitor the cells. Proceed to the next step (passage) when they reach ~80% confluency; this typically occurs every 2–3 d.

v. To passage the ES cells, wash the well that contains the cells with 1 ml of 1× PBS to remove residual proteins. Aspirate the PBS and add 0.25 ml of 0.05% (wt/vol) trypsin for a six-well plate. Rock the plate back and forth to dissociate the cells. Incubate the plate at room temperature for no more than 5 min until all the cells are nearly detached. Do not leave the cells in trypsin for longer than necessary.

vi. Neutralize the trypsin with 1 ml of ES cell medium. Pipette the solution up and down with a P1000 pipette approximately ten times to dissociate the cells into small clumps and single cells.

vii. With a P1000 pipette, add 50 μl of the dissociated ES cells onto a freshly prepared MEF well in 2 ml of fresh ES cell medium to continue growing the cell line at a ~1/25 dilution (roughly, a 1/10 dilution should be ready for passaging in 2 d, a 1/25 dilution in 3 d and a 1/40 dilution in 4 d). The trace amount of trypsin on the first day will not adversely affect the cells.

▲ **CRITICAL STEP** The use of a pipette smaller than P1000 can damage the cells and should be avoided, except to pick colonies or count cells on a hemocytometer. In order to ensure healthy recovery after thawing, ES cells should be passaged at least once before proceeding with the steps below.

viii. If you are electroporating the cells for XEN derivation, passage the cells the day before. If the cells are grown on MEF, MEF-deplete the cells at the same time (see Step 5B(ix)).

ix. MEF-deplete the ES cells. This step is unnecessary if the ES cells are grown in MEF-free conditions. Plate the cells onto a pregelatinized plate in ES cell medium the day before proceeding to XEN derivation. Alternatively, ES cells may be MEF-depleted on the day of derivation by plating them on a pregelatinized 100-mm plate containing 7–10 ml of ES medium (no LIF) for 30 min. After 30 min, the majority of MEFs should have adhered to the pregelatinized plate. Tilt the 100-mm plate to collect the supernatant medium plus ES cells and transfer it into a 15-ml Falcon tube. Do not wash the plate with fresh medium as this may dislodge the attached MEFs.

XEN derivation ● TIMING 15–20 d

6| If you are deriving XEN cells under ES cell conditions, follow option A. If you are deriving XEN cells under TS cell conditions, follow option B. If you are chemically transfecting ES cells with a *Gata6* cDNA plasmid using cationic polymer, follow option C. If you are chemically transfecting ES cells with a *Gata6* cDNA plasmid using lipopolyamine, follow option D. If you are electroporating ES cells with a *Gata6* cDNA plasmid, follow option E. If you are deriving XEN cells from ES cells using growth factors, follow option F.

? TROUBLESHOOTING

(A) XEN cell derivation under ES cell conditions

i. *Day 1*. Thoroughly clean a dissection microscope with 70% (vol/vol) ethanol. By using a mouth-controlled pipette, place one blastocyst per feeder-covered well in the prepared four-well plates (Fig. 4).

▲ **CRITICAL STEP** The plates should be incubated in a 5% CO_2 humidified incubator at 37 °C, except while performing Step 6A(i–iv), which can be performed under ambient conditions.

? TROUBLESHOOTING

ii. *Day 2*. Observe the plates under a microscope. The blastocysts should have hatched from the zona pellucida and attached to the feeder layer (Fig. 4).

iii. *Day 3*. Observe the plates under a microscope. The blastocysts should have started to form an outgrowth (Fig. 4). Carefully aspirate the medium and replace it with fresh ES cell medium.

? TROUBLESHOOTING

iv. *Day 4*. Thoroughly clean a dissection microscope with 70% (vol/vol) ethanol. Dilute 0.25% (wt/vol) trypsin to a sufficient volume of 0.01% (wt/vol) trypsin/1× PBS. Wash the cells with 500 μl of 1× PBS. Aspirate the PBS and add 100 μl of 0.1% (wt/vol) trypsin to each well. Incubate the plates at 37 °C for 5 min. While observing the cells under a cleaned dissection microscope, use a P20 pipette to disaggregate the outgrowth by pipetting up and down several times. Add 400 μl of fresh ES cell medium.

v. *Day 5*. Carefully replace the medium with fresh ES cell medium.

vi. *Days 6–14*. Replace the medium every other day until XEN cell colonies can be observed (Fig. 4).

vii. *Day 15–indefinite*. When 70% confluency is achieved, passage the cells onto a feeder-covered well of a six-well plate. From this point onward, the cells can be cultured in standard XEN medium. Repeat the step, but passage the cells onto a feeder-covered 100-mm dish. Passage the cells two more times. If desired, remove feeders and freeze the XEN cell line as described in Step 7.

(B) XEN cell derivation under TS cell conditions

i. *Day 1*. Thoroughly clean a dissection microscope with 70% (vol/vol) ethanol. By using a mouth-controlled pipette, place one blastocyst per feeder-covered well in the prepared four-well plates (Fig. 4).

▲ **CRITICAL STEP** The plates should be incubated in a 5% CO_2 humidified incubator at 37 °C, except while performing (Step 6B(i–iv)), which can be performed under ambient conditions.

? TROUBLESHOOTING

ii. *Day 2*. Observe the plates under a microscope. The blastocysts should have hatched from the zona pellucida and attached to the MEF feeder layer (Fig. 4).

iii. *Day 3*. Observe the plates under a microscope. The blastocysts should have started to form an outgrowth (Fig. 4). Carefully aspirate the medium and replace it with fresh TS cell medium.

? TROUBLESHOOTING

iv. *Day 4*. Thoroughly clean a dissection microscope by wiping it with 70% (vol/vol) ethanol. Dilute 0.05% (wt/vol) trypsin to a sufficient volume of 0.01% (wt/vol) trypsin/1× PBS. Wash the cells with 500 μl of 1× PBS. Aspirate the PBS and add 100 μl of 0.01% (wt/vol) trypsin to each well. Incubate the plates at 37 °C for 5 min. While observing the cells under a dissection microscope, use a P20 pipette to disaggregate the outgrowth by pipetting up and down several times. Add 400 μl of 30% TS cell medium/70% MEF-conditioned medium.

v. *Day 5*. Carefully replace the medium with fresh 30% TS cell medium/70% MEF-conditioned medium.

vi. *Days 6–14*. Replace the medium every other day until XEN cell colonies are observed (Fig. 4).

vii. *Day 15–indefinite*. When cells reach 70% confluency, passage the cells onto a feeder-covered well of a six-well plate. From this point onward, the cells can be cultured in standard XEN medium. Repeat the step, but passage the cells onto a feeder-covered 100-mm dish. Passage the cells two more times. If desired, remove the feeders and freeze the XEN cell line as described in Step 7.

(C) Chemical transfection of ES cells with a *Gata6* cDNA plasmid using a cationic polymer (e.g., Xfect) for transient or stable transfection

i. *Day 1*. Re-plate MEF-depleted cells onto pregelatinized plates. Cells plated at a density of 7×10^4 per well of a 24-well plate will be ready for transfection ~12 h after plating.

ii. *Day 2*. On the day of transfection, ensure that the ES cells are ~80% confluent. All subsequent volumes are for one well of a 24-well plate and may be scaled up or down according to the manufacturer's recommendations (Clontech).

iii. Prepare two tubes: the first tube contains a 25-µl solution of 1 µg of *Gata6* cDNA plasmid diluted with Xfect reaction buffer in a sterile 1.5-µl Eppendorf tube; the second tube contains 0.5 µl of Xfect polymer (prevortexed) plus 24.5 µl of Xfect reaction buffer in a separate 1.5-µl Eppendorf tube. Use an empty plasmid (i.e., typically the same plasmid containing a constitutively active fluorescent reporter instead of *Gata6*) or sterile dH_2O as negative controls.

iv. Vortex each tube, and then add the polymer solution to the DNA solution (total volume = 50 µl) and vortex again. Incubate the DNA-polymer solution for 10 min at room temperature.

v. To one well of the 24-well plate, add the 50 µl of DNA-polymer solution dropwise. Mix the solution gently by tilting the plate back and forth, and then incubate the plate in a 5% CO_2 humidified incubator at 37 °C for 3 h.

vi. Aspirate the medium containing the DNA-polymer complex from the ES cells, replace it with 0.5 ml of fresh ES cell medium and incubate the plate in a 5% CO_2 humidified incubator at 37 °C overnight.

? TROUBLESHOOTING

vii. *Day 3*. Replace the ES medium in each well with 0.5 ml of XEN medium and incubate the plate overnight in a 5% CO_2 humidified incubator at 37 °C.

viii. *Days 4–10*. If the *Gata6* cDNA plasmid has been engineered to also express a drug-resistance gene, then drug selection can be used to expand successfully transfected cells. Replace with XEN cell medium supplemented with the appropriate drug selection (Table 1). Replace with fresh XEN medium containing drug selection daily for ~10 d. XEN-like cells should appear within 5 d.

ix. *Day 11–indefinite*. Enzymatically passage the *Gata6* cDNA–transfected cells with 0.05% (wt/vol) trypsin onto a pregelatinized 100-mm dish. Continue selection with drugs in order to establish a XEN cell line. Collect a fraction of the cells for genotyping and freeze the remaining XEN cells as described in Step 7.

(D) Chemical transfection of ES cells with a *Gata6*-cDNA plasmid using lipopolyamine (e.g., Lipofectamine 2000) for transient or stable transfection

i. *Day 1*. Re-plate MEF-depleted cells onto pregelatinized plates. Cells plated at a density of 7×10^4 per well of a 24-well plate will be ready for transfection ~12 h after plating.

ii. *Day 2*. On the day of transfection, ensure that the ES cells are ~80% confluent. All subsequent volumes are for one well of a 24-well plate and may be scaled up or down according to the manufacturer's recommendations (Invitrogen).

iii. Prepare two tubes: the first tube contains a 50-μl solution containing 1 μg of Gata6 cDNA plasmid diluted with Opti-MEM I in a sterile 1.5-μl centrifuge tube; the second tube contains 4.5 μl of Lipofectamine 2000 plus 45.5 μl of Opti-MEM I in a separate 1.5-μl centrifuge tube. Use an empty plasmid (i.e., typically the same plasmid containing a constitutively active fluorescent reporter instead of *Gata6*) or sterile dH_2O as negative controls. Incubate the tubes for 5 min at room temperature.

iv. Gently mix the diluted plasmids (*Gata6* or control) with the diluted Lipofectamine reagent (total volume = 100 μl) and incubate the tubes for 20 min at room temperature to allow for the formation of DNA-liposome complexes. During the 20-min incubation period, replace the medium of the plated cells with 0.5 ml of fresh ES cell medium without antibiotics.

v. To one well of the 24-well cell culture dish, add 100 μl of DNA-liposome complex dropwise. Mix gently by tilting the plate back and forth, and then incubate the plate overnight in a 5% CO_2 humidified incubator at 37 °C.

? TROUBLESHOOTING

vi. *Day 3*. Replace the ES medium in each well with 0.5 ml of XEN medium and incubate the plate overnight in a 5% CO_2 humidified incubator at 37 °C.

vii. *Days 4–10*. If the *Gata6*-cDNA plasmid has been engineered to also express a drug-resistance gene, then drug selection can be used to expand successfully transfected cells. Replace the medium with XEN cell medium supplemented with the appropriate drug selection (Table 1). Replace with fresh XEN medium containing drug selection daily for ~10 d. XEN-like cells should appear within 5 d.

viii. *Day 11–indefinite*. Enzymatically passage the *Gata6* cDNA–transfected cells with 0.05% (wt/vol) trypsin onto a pregelatinized 100-mm dish. Continue selection with drugs to establish a XEN cell line. Collect a fraction of the cells for genotyping and freeze the remaining XEN cells as described in Step 7.

(E) Electroporation of ES cells with a *Gata6* cDNA plasmid for transient or stable transfection

i. *Day 1*. On the day of electroporation, ensure that the ES cells are ~80% confluent in one 100-mm tissue culture plate. Aspirate the medium from the 100-mm culture plate and wash the cells with 5 ml of PBS.

ii. Aspirate the PBS and trypsinize the cells with 1 ml of 0.05% (wt/vol) trypsin. Quench the trypsinized cells with 3 ml of MEF medium.

iii. Transfer the ES cells to a 15-ml Falcon tube and pellet the cells at 200*g* for 4 min at room temperature. Aspirate the MEF medium and resuspend the ES cell pellet with 5 ml of PBS.

iv. Pellet the cells at 200*g* for 4 min at room temperature. Aspirate the PBS and resuspend the ES cells in 0.7 ml of PBS.

v. Gently mix the linearized *Gata6* cDNA sample with the resuspended cells and incubate the mixture for 5 min at room temperature.

vi. Transfer the DNA-cell mixture to a 4-mm electroporation cuvette. Electroporate the mixture at 500 μF/230 V. The time constant should be between 6 and 8 ms.

vii. Allow the ES cells to recover in the cuvette for 5 min at room temperature and then transfer the cells into 10 ml of ES cell medium; plate the entire volume onto a MEF-coated 100-mm plate. Mix gently by tilting the plate back and forth, and then incubate the plate overnight at 37 °C.

viii. *Day 2*. Exchange the medium with 10 ml of standard ES cell medium and incubate the plate overnight in a 5% CO_2 humidified incubator at 37 °C.

ix. *Days 3–9*. If the *Gata6*-cDNA plasmid has been engineered to also express a drug-resistance gene, then drug selection can be used to expand successfully transfected cells. Exchange the medium with 10 ml of standard XEN cell medium supplemented with the appropriate drug selection (Table 1). Replace the medium with fresh XEN medium containing drug selection daily for ~10 d. XEN-like cells should appear within 5 d.

? TROUBLESHOOTING

x. *Day 10–indefinite*. Enzymatically passage the *Gata6* cDNA–transfected cells with 0.05% (wt/vol) trypsin onto a pregelatinized 100-mm dish. Continue selection with drugs to establish a XEN cell line. Collect a fraction of the cells for genotyping and freeze the remaining XEN cells as described in Step 7.

(F) cXEN cell derivation from ES cells

i. *Day 1*. Enzymatically passage MEF-depleted ES cells with 0.05% (wt/vol) trypsin. Inhibit the trypsin with XEN medium.

ii. Centrifuge the ES cells at 200*g* for 4 min at room temperature and aspirate the supernatant. Be stringent in aspirating (without touching the pellet). This step removes ES medium, which is important as lingering LIF potentially inhibits cXEN derivation.

iii. Resuspend the pellet in ~5 ml of standard XEN medium. Mix the tube well and immediately remove two 20-μl aliquots to count MEF-depleted ES cells on a hemocytometer. Take the average (n = 3) number of cells in one big square (16 little squares), multiply by 10^4 and multiply by the number of milliliters of medium, which will be equal to the total number of cells.

iv. Remove the gelatin from the six-well cXEN derivation plate and add fresh standard XEN medium. Add the appropriate volume of trypsinized ES cells to start the cXEN derivation procedure for a final density of 1×10^4 cells per cm^2 (i.e., 9.6×10^4 cells per well of a six-well plate; Fig. 5). Incubate the plate overnight at 37 °C in standard XEN medium. The cell density is an important determinant of differentiation efficiency, and as different ES cell lines proliferate at different rates, we recommend determining the optimal density for each line.

v. *Day 2*. Twenty-four hours after the initial plating, aspirate the standard XEN medium and replace it with 2 ml of cXEN derivation medium per well (Fig. 5). Incubate the plate overnight in a 5% CO_2 humidified incubator at 37 °C.

vi. *Day 3*. Aspirate the cXEN derivation medium and replace it with fresh cXEN derivation medium. Incubate the plate overnight in a 5% CO_2 humidified incubator at 37 °C.

vii. *Day 4*. Enzymatically passage the cells with 0.05% (wt/vol) trypsin. Quench the trypsin with 0.5 ml of standard XEN medium. Aspirate the MEF medium in a MEF-coated six-well plate and replace it with 2 ml of standard XEN medium per well. Transfer the entire contents of the differentiated cells and distribute the cells evenly into the well of the MEFcoated plate (1:1 dilution; Fig. 5).

viii. *Days 5–11*. Aspirate the medium from the cXEN cells and replace it with fresh standard XEN medium every day or every other day depending on confluency. XEN-like cells with stellate and refractile morphology will emerge ~5 d after the first passage on MEFs; however, the well will also contain cells of different morphologies. Use standard XEN medium hereafter. During the first (and sometimes second) passage, the cXEN cells recover better when MEFs are present, although they are not a requirement. FGF2/FGF4 and heparin can also be added during the derivation, but they too are not a requirement as long as endogenous *Fgf4* is intact.

ix. *Days 12–19*. Manually pick XEN-like cells using a 20-μl pipette under a microscope to facilitate the ES to cXEN cell derivation (Fig. 5). Depending on the density, place the isolated cells in a MEF-coated (low density of XEN-like cells) or pregelatinized plate (high density of XEN-like cells) in XEN medium and continue feeding with standard XEN medium.

? TROUBLESHOOTING

x. *Day 20–indefinite*. Once the cells reach 70–90% confluency, passage the cells enzymatically with 0.05% (wt/vol) trypsin and plate them onto a pregelatinized or MEF-coated 100-mm plate or several wells of a six-well plate. The majority of cells should morphologically resemble XEN cells. If there are any contaminating cells that are not XEN-like cells, aspirate, pick away or reciprocally pick the XEN-like cells to enrich for this population in a new plate or well. Passage two more times onto pregelatinized plate or well and freeze the cXEN cell line (Step 7).

DNA extraction and freezing cells ● TIMING 1–2 d

7| cXEN or XEN cells can be frozen and thawed using conventional stem cell freeze-thaw protocols in freezing medium. Passage as above and resuspend the trypsinized cell pellet in prechilled (4 °C) freezing medium as described in option A. If you wish to extract DNA from *Gata6* cDNA–targeted ES cells, there are a number of different extraction protocols that may be used. Option B describes the Promega SV Wizard purification system.

(A) Freezing cells

i. Transfer the resuspended cells into prelabeled cryotube vials.

ii. Quickly transfer the cryotube vials to − 80 °C freezer overnight covered with Styrofoam.

■ **PAUSE POINT** For long-term storage, keep cells frozen in a liquid nitrogen dewar.

(B) Lysing cells for genomic DNA extraction

i. Enzymatically passage cells as above and resuspend the cell pellet in the Promega Wizard SV lysis buffer according to the manufacturer’s instructions, and then mix the cell lysate by pipetting.

■ **PAUSE POINT** The cell lysates can be frozen at − 80 °C until needed.

ii. Transfer each sample lysate from the cell culture plate to a separate Wizard SV minicolumn assembly.

iii. Spin the assembly at $16.1 \times 10^3 g$ for 3 min at room temperature.

iv. Remove the minicolumn from the assembly and discard the liquid in the collection tube. Replace the minicolumn into the collection tube.

v. Add 650 µl of Wizard SV wash solution (with 95% (vol/vol) ethanol added) to each assembly. Centrifuge the tube at $16.1 \times 10^3 g$ for 1 min at room temperature. Discard the liquid from the collection tube.

vi. Repeat Step 7B(v) for a total of four washes.

vii. Discard the liquid from the collection tube and reassemble the minicolumn assembly. Centrifuge for 2 min at $16.1 \times 10^3 g$ at room temperature to dry the binding matrix.

viii. Transfer the Wizard SV minicolumn to a new 1.5-ml tube. Add 50 µl of room-temperature, nuclease-free water. Incubate the minicolumn for 2 min at room temperature.

ix. Centrifuge the minicolumn at $16.1 \times 10^3 g$ for 1 min at room temperature.

x. Remove the minicolumn and store the purified DNA at − 20 or − 70 °C for several months or years, respectively.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 2.

● TIMING

Steps 1–5, preparation of MEF feeders: 15–30 min

Step 6, XEN derivation: 15–20 d

Step 7, DNA extraction and freezing cells: 1–2 d

Box 1, preparation of *Gata6* cDNA for transfection or electroporation: ~2 d

ANTICIPATED RESULTS

We have used the protocols described here to establish XEN cell lines from either mouse blastocysts or ES cells. XEN cell lines exhibit characteristic heterogeneous morphology with both highly refractile phase-bright and epithelial-like cells (Figs. 2, 4 and 5). XEN cells can be distinguished from mouse ES cells as the latter form dome-shaped clusters of cells with characteristic high nuclear-to-cytoplasmic ratio[4,5], whereas the former grow as individual cells and the nucleus of each cell is not clearly distinguishable (compare Fig. 2). XEN cells are also distinct from TS cells that grow as epithelial colonies comprising cells with distinct nuclei and can differentiate into multinucleated trophoblast giant cells *in vitro*[2] (compare Fig. 2). It was previously demonstrated that XEN cells oscillate between these two morphologies[3]. All XEN cell lines retain the expression of key XEN-associated genes including the GATA transcription factor Gata4, the SOX factor Sox7 and Disabled homolog 2 Dab2 (Supplementary Fig. 1). Notably, these genes are not homogeneously expressed in all cells within a XEN cell line (Supplementary Fig. 1), and it remains unclear whether heterogeneity in morphology and gene expression reflects a fixed or oscillating heterogeneity representing distinct XEN cell types or substrates present in culture. Notably, mouse XEN cells usually lack the expression of ES cell–associated genes including

octamer-binding transcription factor Oct4 (Supplementary Fig. 1) and Nanog. XEN cells self-renew indefinitely in culture in the absence of exogenous growth factors such as FGF2 (refs. 13,16). Moreover, XEN cells can be directed to differentiate into α-fetoprotein (Afp)-expressing visceral endoderm-like cells with the addition of BMP4 (refs. 18,19) and are committed to primitive endoderm-derived lineages in chimeric embryos[3,34]. Although not reported in the literature, in our own experience the karyotype of XEN cells can change over time and cell lines can acquire karyotypic anomalies with extended passage in culture (N.S. and A.-K.H., unpublished observations). It is therefore preferable to work with cells that are of as low a passage as possible. It should be noted that even clonal XEN cell lines exhibit some degree of variability, for example, in the ratio of cells exhibiting different morphologies and/or in the expression of molecular markers. It is currently not clear whether this inherent variability reflects the cell of origin within an embryo or ES cell culture, cell culture history or an unidentified stochastic or deterministic factor. Furthermore, although there is no 'gold standard' XEN cell line that is used across laboratories, as a point of reference, especially when first establishing methods for XEN cell culture within a laboratory, it is advisable to obtain an established and characterized XEN cell line from another laboratory. In contrast to ES cells, no clear strain biases have been reported for the derivation of XEN cells from embryos or conversion from ES cells. This is likely because the protocols for XEN cell derivation, as provided here, are very efficient as compared with most non-inhibitor protocols for ES cell derivation. Notably, even though XEN cells are a relatively new stem cell type, which can be used in a number of applications, in the future, it will be important to compare the differentiation efficiency and molecular identity of XEN cells derived under distinct culture conditions to determine whether there may be biases in potential and/or differences in gene expression. In all, XEN cells are emerging as a useful stem cell model for understanding the convergence of signaling and transcriptional control during XEN cell–fate specification and differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1 | Preparation of *Gata6* cDNA for transfection or electroporation ● TIMING ~2 d

1. In order to ensure sufficient quantity of plasmid DNA, a large-scale preparation of *Gata6* cDNA plasmid by maxiprep is required for transfection. A circular *Gata6* cDNA plasmid is generally used for chemical-based transfection, whereas for electroporation, the plasmid should be linearized using a unique restriction enzyme at a non-essential region, which will not affect the function of the gene and drug selection.
2. If linearized DNA is required then cut one time with a unique restriction enzyme that is in a region of the plasmid that will not affect the function. Linearize 100 μg of *Gata6* cDNA plasmid by restriction enzyme digestion overnight at the optimal incubation temperature. Run a small aliquot on a 0.8% (wt/vol) agarose gel to ensure linearization.
3. For high-purity plasmid preparation, precipitate the linearized *Gata6* cDNA with a 1/10-volume of salt (3 M sodium acetate) and 2.5 volumes of 100% ethanol; store at − 20 °C overnight.
4. The next day, spin the DNA at $16.1 \times 10^3 g$ for 5 min at 4 °C and wash the pellet once with 500 μl of 70% (vol/vol) ethanol.
5. Remove the supernatant and allow any remaining ethanol to evaporate in the hood for several min.

   ▲ **CRITICAL STEP** To ensure sterility, work in a sterile tissue culture hood for this and subsequent steps.
6. Resuspend the DNA pellet in 100 μl of sterile PBS without Mg^{2+} or Ca^{2+} (a final DNA concentration of 1 μg μl^{-1}).

   ▲ **CRITICAL STEP** The presence of salts could be detrimental to transfection efficiency and interfere with electroporation.

   ■ **PAUSE POINT** Plasmid DNA preparation can be done any time before transfection. The resuspended DNA can be kept at − 20 °C for several months.

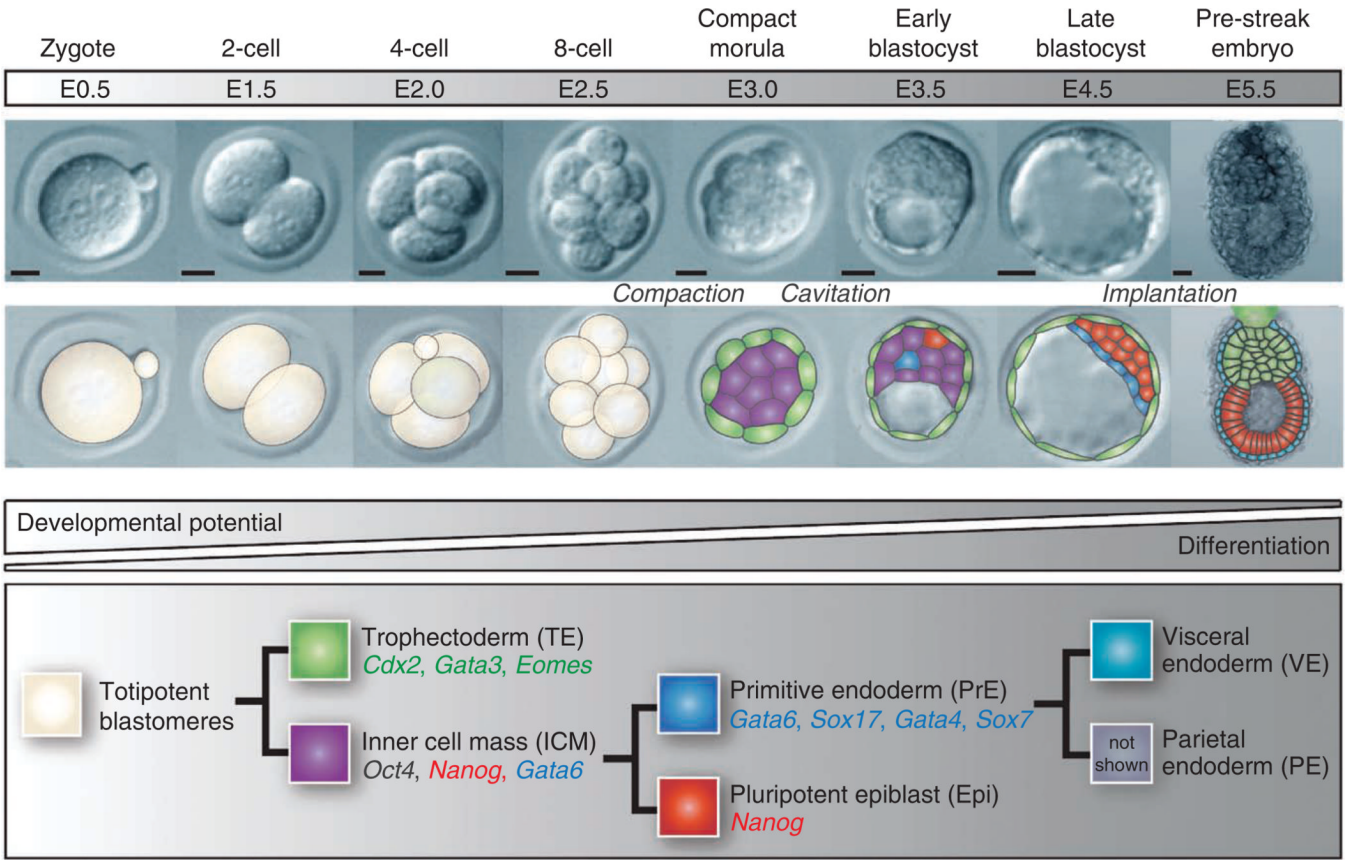

Figure 1.

Overview of early embryonic development. Proper lineage segregation before implantation is ensured by two cell-fate decisions, with the first giving rise to trophectoderm and inner cell mass, and the second leading to the allocation of primitive endoderm and epiblast. Lineage-associated gene expression is noted below each cell type. After implantation, the PrE differentiates into visceral and parietal endoderm. E: embryonic day.
Scale bars, 50 μm.

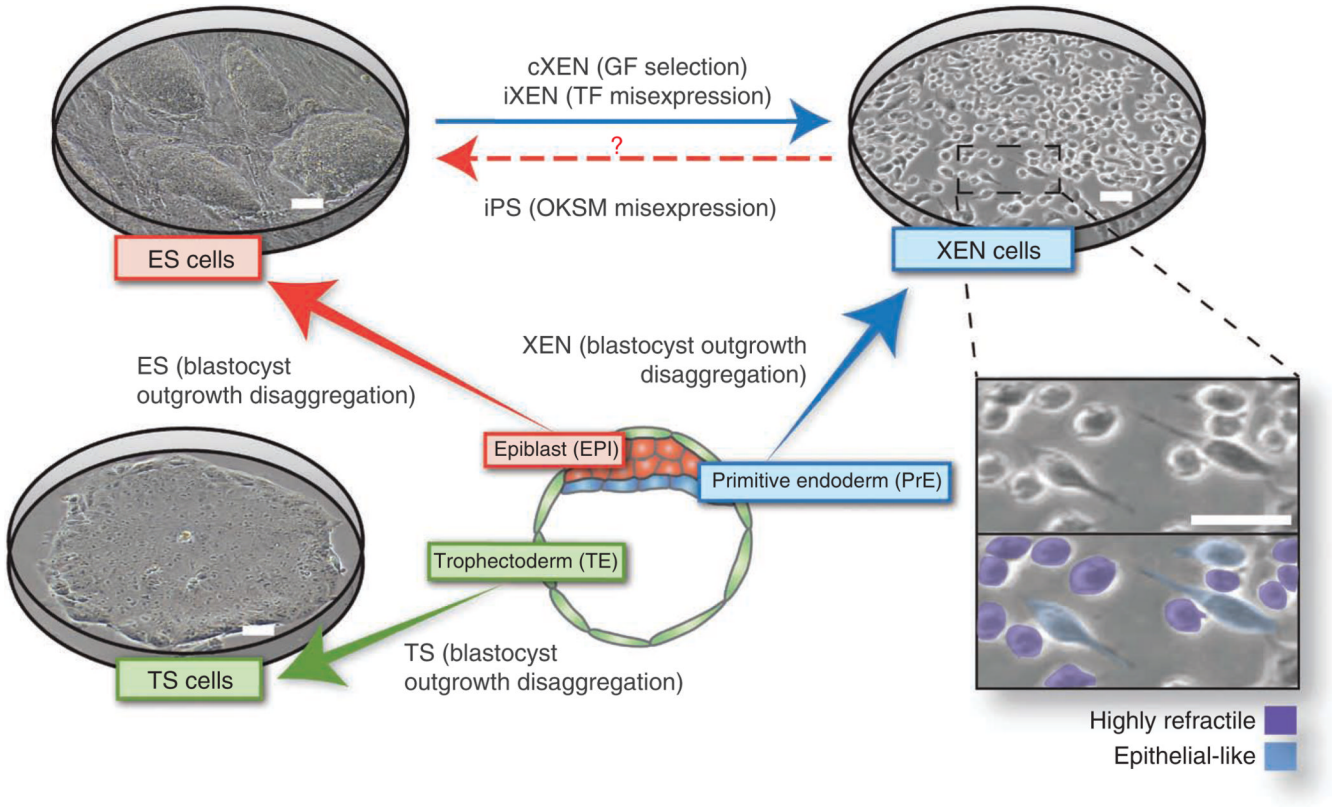

Figure 2.

Stem cell types that can be derived and propagated in culture representing the three blastocyst lineages. Embryonic stem (ES) cells represent the epiblast, trophoblast stem (TS) cells represent the trophectoderm and extraembryonic endoderm (XEN) cells represent the primitive endoderm cell lineage. Heterogeneities in XEN cell morphology are indicated: highly refractile phase-bright and epithelial-like. Cognate embryo– derived stem cells retain the expression of key lineage-associated genes. GF, growth factor; iPS, induced pluripotent stem; OKSM, Oct4, Sox2, Klf4 and c-Myc. Scale bars, 100 μm.

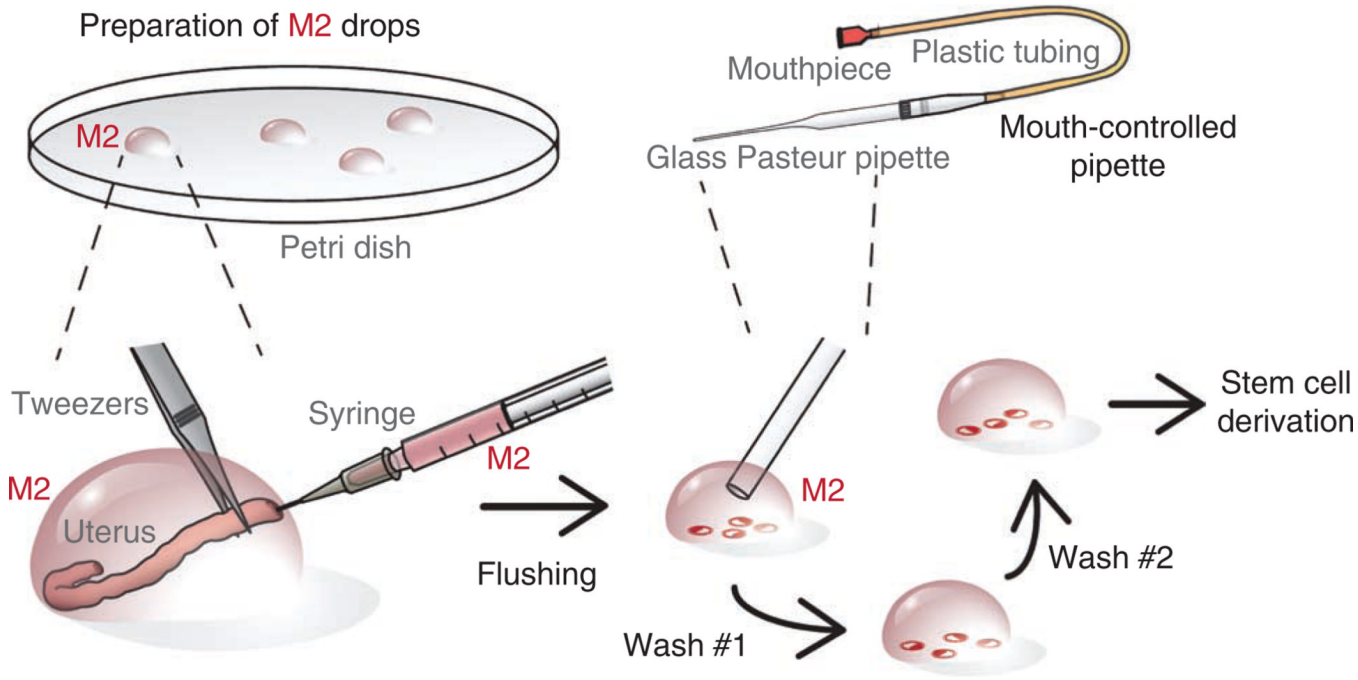

Figure 3.

Recovery of blastocyst-stage embryos from uteri of adult female mice. Several drops of M2 medium are prepared on the lid of a 100-mm dish. The dissected and cleaned uterine horn of a pregnant female (3.5 d.p.c.) is placed in one drop. While securing the uterine horn with forceps, the needle of a 1-ml syringe is inserted and the uterine horn is flushed with ~0.2 ml of M2 medium. The flushed blastocysts are located under high magnification and transferred to a fresh drop of M2 medium using a mouth-controlled pipette. This transfer is repeated at least twice to wash away debris, lipid drops and blood cells.

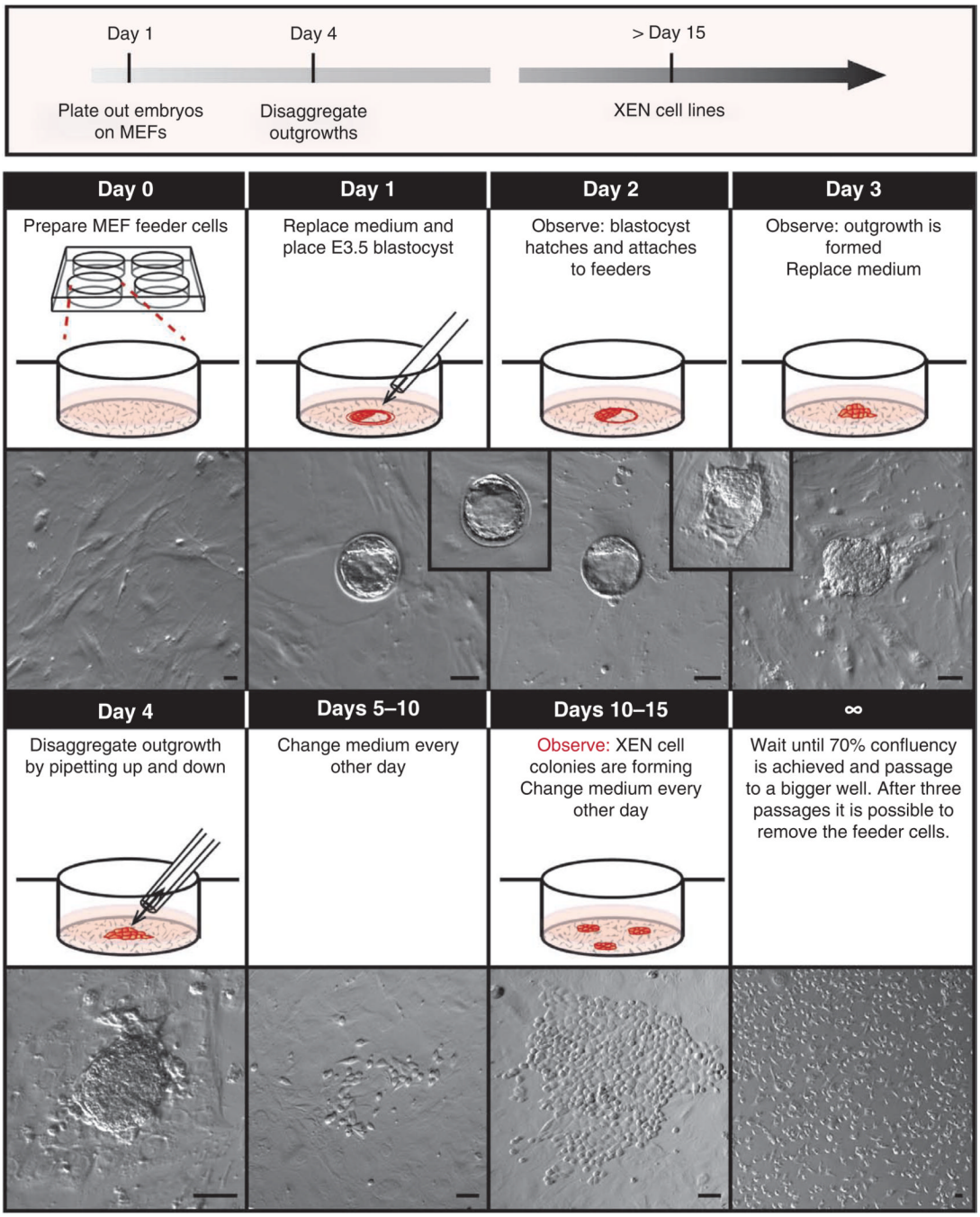

Figure 4.

Timeline for XEN cell derivation from mouse blastocysts. Protocol for the derivation of XEN cells from blastocysts. Day 0: feeder cell–coated four-well plates are prepared. Day 1: medium is replaced and a freshly flushed E3.5 blastocyst is placed in the center of the well. Day 2: the blastocyst hatches and attaches to the feeder cells. Day 3: the blastocyst has formed an outgrowth. Medium is replaced. Day 4: the prominent outgrowth is trypsinized and disaggregated with a P20 pipette. Days 5–10: the medium is replaced every other day and the plate is observed daily. Days 10–15: XEN-like cells with stellate and refractile morphology will emerge. The medium is replaced every other day and the plate is observed daily. Day 15 and onward: After 70% confluency is attained, the XEN cells are passaged

onto a well in a six-well plate and subsequently into a 100-mm dish. After three more passages they can be MEF-depleted and the XEN cell line can be frozen. Scale bars, 100 μm.

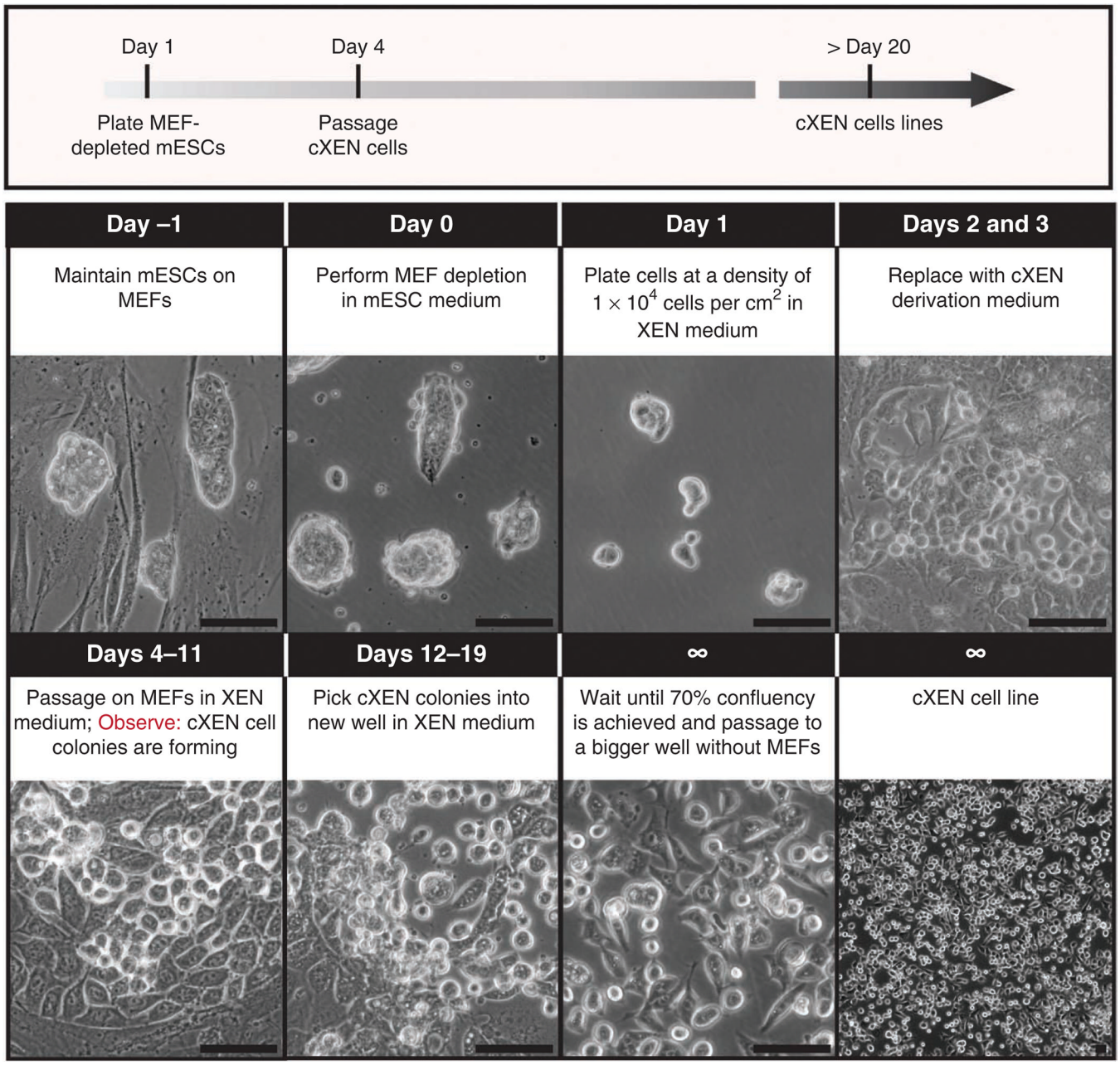

Figure 5.

Timeline for cXEN cell derivation from mouse ES cells. Protocol for the conversion of ES cells to cXEN cells using growth factors. Day − 1: ES cells are maintained in ES medium on MEFs. Day 0: ES cells are passaged onto a pregelatinized plate for MEF depletion. Day 1: ES cells are enzymatically passaged with 0.05% (wt/vol) trypsin and plated at a density of 1 × 10^4 cells per cm^2 in standard XEN medium. Days 2 and 3: the medium is replaced with cXEN derivation medium daily (0.01–10 μM retinoic acid plus 10 ng ml^{-1} activin A). Days 4–11: cells are enzymatically passaged onto MEFs in XEN medium. The medium is replaced every day or every other day depending on confluency. XEN-like cells with stellate and refractile morphology will emerge within ~5 d. Days 12–19: XEN-like cells are picked manually and placed in a MEF-coated or pregelatinized plate in XEN medium. They are

passaged two more times onto pregelatinized plates and the cXEN cell line is frozen. Scale bars, 100 μm.

Table 1

Drug selection.

Marker	Gene product	Selection method
Neo	Aminoglycoside phosphotransferase; *neo* gene from the bacterial transposon *Tn5*	Select cells in G418 (0.1–1.0 mg ml^{-1}), an aminoglycoside that blocks protein synthesis and is similar to kanamycin
Hyg	Hygromycin-B-transferase; *hyg* gene from *Escherichia coli*	Select cells in hygromycin-B (10–300 mg ml^{-1}), an aminocyclitol that inhibits protein synthesis
Pac/Puro	Puromycin-N-acetyl transferase; *pac* gene from *Streptomyces alboniger*	Select cells in puromycin (0.5–5 mg ml^{-1}), an antibiotic that inhibits protein synthesis
Zeo	Bleomycin-binding protein; a *zeo* gene (a.k.a. blaR) is located on the bacterial transposon *Tn5*	Select cells in bleomycin or commercially available Zeo (50–500 mg ml^{-1}), an antibiotic that binds DNA and blocks RNA synthesis

Table 2

Troubleshooting table.

Step	Problem	Possible reason	Solution
1–6	Contamination	Poor sterile technique	Ensure that all surfaces are thoroughly cleaned with 70% (vol/vol) ethanol before use and observe proper tissue culture technique
5A(iv)	Finding blastocysts	Lipid drops, blood cells and debris are impeding the view	Adjust magnification and mirror angle to identify blastocysts by their distinctive surrounding zona pellucida
6A(i), 6B(i)	Differentiation	Blastocyst misattachment	Blastocysts should be placed in the center of the well to avoid attachment to the sides of the well
6A(iii), 6B(iii)	Small outgrowth	Insufficient time to develop	If no prominent outgrowth can be observed, wait an additional day before proceeding with the next steps
6	Low cell density	Low viability and/or contamination	It is imperative to closely observe the cells daily. XEN cell colonies can be observed as early as 10 d but might take up to 15 d to appear. Check for possible contamination
6B	TS cells outcompeting XEN cells	Addition of FGF and heparin; dissociation of the outgrowth	TS cells are more likely to outcompete the XEN cells later on. If this is observed, desist from adding FGF and heparin to the TS cell medium, and dissociate the outgrowth less rigorously. In some cases, it can be beneficial to entirely refrain from dissociating the outgrowth
6C, 6D, 6E	Insufficient expression of exogenous *Gata6*	*CMV* promoter may be silenced in ES cells	We recommend using a plasmid with an *EF1A* or *CAGG* promoter to ensure robust expression
6D	Low transfection efficiency	Serum in the medium may interfere with DNA-liposome complex formation	Opti-MEM I can be used during the overnight incubation instead of ES cell medium without penicillin or streptomycin to improve the transfection efficiency; however, this may compromise ES cell viability