Derivation of mouse extraembryonic endoderm stem cell lines, and exclusive transmission of the embryonic stem cell-derived genome through the mouse germline

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Contents

List of Abbreviations

2i	Two inhibitor (PD0325901 and Chir99021)
3D	Three-dimensional
AFP	Alpha-fetoprotein
AVE	Anterior visceral endoderm
BMP	Bone morphogenetic protein
BMP4	Bone morphogenetic protein 4
CM	MEF-conditioned medium
DNA	Deoxyribonucleic acid
EDS	Embryonic-derived stem
emVE	Embryonic visceral endoderm
exVE	Extraembryonic visceral endoderm
EPI	Epiblast
EpiSC	Epiblast stem cell
EPSC	Expanded potential stem cell
EPSCM	Expanded potential stem cell medium
Erk	Extracellular signal–regulated kinase
ES	Embryonic stem
Esrrb	Estrogen related receptor beta
ExE	Extraembryonic ectoderm
ExEn	Extraembryonic endoderm
FGF4	Fibroblast growth factor 4
F4H	25 ng/ml FGF4 and 1 mg/ml heparin
GFP	Green fluorescent protein
GSK	Glycogen synthase kinase
hESC	Human embryonic stem cell
ICM	Inner cell mass
iPSC	Induced pluripotent stem cell
JAK-STAT	Janus kinase/signal transducers and activators of transcription
KSR	Knockout serum replacement
LIF	Leukemia inhibitory factor
MAPC	Multipotent adult progenitor cell

MEF	Mouse embryonic fibroblast
MuERV-L	Murine endogenous retrovirus with leucine tRNA primer
nEnd	Naïve extraembryonic endodermal (cell)
OSKM	OCT4, SOX2, KLF4 and MYC
PASE	Postimplantation amniotic sac embryoid
PDGF	Platelet-derived growth factor
PDGFRA	Platelet-derived growth factors receptor alpha
PE	Parietal endoderm
PECAM1	Platelet endothelial cell adhesion molecule-1
Post-XEN	XEN cell derived from a post-implantation embryo
PrE	Primitive endoderm
Pre-XEN	XEN cell derived from a pre-implantation embryo
pXEN	Primitive extraembryonic endoderm stem
rHypoSC	Rat hypoblast stem cell
rMAPC	Rat multipotent adult progenitor cell
RA	Retinoic acid
SSEA1	Stage-specific embryonic antigen-1
Sox	Sry-related high-mobility group box
TE	Trophectoderm
TGF	Transforming growth factor
Wnt	Wingless-type MMTV integration site family
TS	Trophoblast stem
VE	Visceral endoderm
VEGF	Vascular endothelial growth factor
XEN	Extraembryonic endoderm stem
XEN-P	Extraembryonic endoderm precursor

Deutsche Zusammenfassung

Die Analyse früher Entwicklungsstadien von Säugetierembryonen und daraus gewonnener Stammzelllinien kann entscheidende Erkenntnisse im Bereich der Reproduktionsbiologie und der regenerativen Medizin hervorbringen. Dabei spielt die Maus, als geeignetes Modellsystem für die Übertragbarkeit auf den Menschen eine wichtige Rolle, in erster Linie weil die Blastozysten der Maus verglichen mit menschliche Blastozysten eine morphologische Ähnlichkeit aufweisen. Humane embryonale Stammzelllinien haben großes Potential für die Anwendung in der regenerativen Medizin und vergleichend dazu wurde Gen-Targeting in embryonalen Stammzellen verwendet, um tausende neuer Mausstämme zu generieren. Die Gewinnung embryonaler Stammzellen erfolgt im Blastozystenstadium, diese können dann nach Injektion in eine andere Blastozyste zur Entwicklung aller Gewebearten, einschließlich der Keimbahngewebe, beitragen (Martin, 1981; Evans and Kaufman 1981).

Ursache einer Fehlgeburt können vor allem Defekte in der Entwicklung des Trophoblasten und des primitive Entoderms (PrE) sein, dabei sind ca. 5 % der Paare betroffen die versuchen ein Kind zu bekommen (Stephenson and Kutteh, 2007). Eine Untersuchung dieser Zelllinien im Mausmodell könnte weitere Erkenntnisse für die Gründe einer Fehlentwicklung liefern. Trophoblasten Stammzelllinien können aus den Blastozysten der Maus und dem extraembryonalen Ektoderm von bereits implantieren Embryonen gewonnen werden (Tanaka et al., 1998). Diese Zelllinien geben Aufschluss über die Entwicklung des Trophoblasten, fördern die Entwicklung der Plazenta und sind gleichzeitig ein gutes Modellsystem um die Implantation des Embryos im Uterus näher zu untersuchen. Zellen des primitive Entoderms (PrE) beeinflussen das im Dottersack vorhandene extraembryonale Entoderm, welches dort als “frühe Plazenta” fungiert und für die Versorgung des Embryos mit Nährstoffen zuständig ist (Cross et al., 1994). Des Weiteren besitzt das Entoderm einen induktiven Einfluss auf die Bildung von anterioren Strukturen und die Bildung von Endothelzellen sowie Blutinseln (Byrd et al., 2002).

Extraembryonale Endodermstammzellen (XEN Zellen) können aus Blastozysten gewonnen und in embryonale Stammzellen (ES-Zellen) umgewandelt werden (Fujikura et al., 2002; Kunath et al., 2005). Es war jedoch nicht bekannt, ob XEN-Zellen auch aus Postimplantations-Embryonen gewonnen werden können. XEN-Zellen tragen in vivo zur Entwicklung des Darmendoderms bei (Kwon et al., 2008; Viotti et al., 2014) und könnten

als alternative, selbsterneuernde Quelle für extraembryonale Endoderm-abgeleitete Zellen dienen, die zur Herstellung von Geweben für die regenerative Medizin verwendet werden könnten (Niakan et al., 2013).

In der Embryogenese der Maus zeigt sich an Tag E3.0 eine kompakte Morula die sich allmählich in das Trophektoderm (TE) differenziert, welches wiederum den Embryonalknoten (“innere Zellmasse”) umschließt (Johnson and Ziomek, 1981). Ein wichtiger Schritt im Rahmen der Entwicklung findet an Tag E3.5 statt, in diesem Zeitraum gehen aus dem Embryonalknoten der pluripotente Epiblast und das primitive Entoderm hervor. Im späten Blastozystenstadium an Tag E4.5 liegt das PrE als Zellschicht entlang der Oberfläche der Blastocoel-Höhle. Aus dem Epiblast entwickeln sich im weiteren Verlauf der Embryo, das Amnion und das extraembryonale Mesoderm des Dottersacks. Die Zellen des Trophektoderm führen zur Entwicklung der Plazenta. Das PrE differenziert sich im Zuge der Weiterentwicklung in das viszerale Entoderm (VE) und das parietale Entoderm (PE) des Dottersacks (Chazaud et al., 2006; Gardner and Rossant, 1979; Plusa et al., 2008). VE umgibt den Epiblast und extraembryonisches Ektoderm (ExE). PE-Zellen wandern entlang der inneren Oberfläche von TE und sezernieren zusammen mit Trophoblasten-Riesenzellen Basalmembranproteine, um die Reichert-Membran zu bilden (Hogan et al., 1980). Die Reichert-Membran besteht aus Basalmembranproteinen, einschließlich Kollagenen und Lamininen, die zwischen den parietalen Endoderm- und Trophoblastzellen liegen. Diese Membran wirkt als ein Filter, der dem Embryo den Zugang zu Nährstoffen ermöglicht, während er eine Barriere zu den Zellen der Mutter bildet (Gardner, 1983).

Insgesamt gibt es drie Typen von Stammzelllinien die aus den ersten drei Zellentwicklungsstadien des Mausembryos gewonnen werden können. Dabei handelt es sich um embryonale Stammzellen, Trophoblasten Stammzellen und extraembryonale Entoderm-Stammzellen (XEN-Zellen) die für die Untersuchung von Zellschicksalsentscheidungen bereits verwendet wurden. Embryonale Stammzellen können aus dem in den Blastozysten vorhandenen Epiblast, oder auch aus schon bereits in die Gebärmutter implantierten Embryonen gewonnen werden (Evans and Kaufman, 1981; Bao et al., 2009). Die Isolierung von Trophoblasten Stammzellen erfolgt aus dem Throphoblasten selbst, als auch aus implantierten Embryonen, außerdem können auch durch Weiterentwicklung embryonaler Stammzellen Throphoblastenzelllinien gewonnen werden (Tanaka et al., 1998; Lu et al., 2008). Extraembryonale Entoderm-Stammzelllen

werden von Zellen des PrE, durch die Umwandlung von ES-Zellen, oder durch die Reprogrammierung von Fibroblasten unter der Verwendung der Transkriptionsfaktoren *Oct3/4, Sox2, Klf4* und *c-Myc* (OKSM) abgeleitet (Kunath et al., 2005; Fujikura et al., 2002; Parenti et al., 2016). ES-Zellen können sich in totipotent-ähnliche Zellen umwandeln, die das Potenzial haben, sich in drei Zelllinien zu differenzieren (Macfarlan et al., 2012). EDS-Zellen sind in vitro in der Lage, Strukturen aufzubauen, die dem Postimplantation-Embryo bzw. der Blastozyste ähneln (Harrison et al., 2017; Kime et al., 2018; Rivron et al., 2018).

Zahlreiche Veröffentlichungen haben gezeigt, dass Sox17, PDGFRA und GATA6 für die Ableitung und Erhaltung von XEN Zellen notwendig sind (Cho et al., 2012; Artus et al., 2010; Niakan et al., 2010). XEN-Zelllinien werden typischerweise zur Erforschung der biologischen Eigenschaften dieser Zellen verwendet, dienen aber auch dazu, Differenzierungseigenschaften von PrE in VE und PE zu untersuchen. Extraembryonale Entodermzellen tragen zur Bildung des Darmentoderms bei und stellen sich als eine Alternative für sich selbst erneuernde Quelle von Entoderm abstammenden Zellen dar (Kwon et al., 2008; Viotti et al., 2014). Eine Verwendung von XEN-Zellen für die Produktion von Geweben für die regenerative Medizin konnte bereits gezeigt werden (Niakan et al., 2013).

PDGFRA und Sox17 sind wichtig, um die PrE-Zelllinie zu bilden. Die Deletion von PDGFRA oder Sox17 verringert die Anzahl von PrE-Zellen (Artus et al., 2011, 2013). PDGFRA-mutierte Embryonen können sich noch bis zur Geburt entwickeln, jedoch mit schweren Defekten, und sterben bald nach der Geburt (Ogura et al., 1998). Sox17-mutierte Embryonen können nach dem E8.0-Stadium nicht überleben (Artus et al., 2013). Es gibt Berichte, dass PDGFRA und Sox17 essentiell für die Ableitung von XEN-Zelllinien sind (Artus et al., 2010; Niakan et al., 2010; Cho et al., 2012). Ich stellte die Hypothese auf, dass XEN-Zelllinien von PDGFRA-mutierten Embryonen und ES-Zellen abgeleitet werden können, da die verbleibende PrE immer noch die Fähigkeit besitzt, die Entwicklung von Embryonen zu unterstützen.

Das Gene-Targeting in ES-Zellen ist nach wie vor die beste Methode, um komplexe Mutationen in die Maus-Keimbahn einzuführen. Aber die Übertragung des ES-Zell-Genoms durch die Keimbahn von Chimären zu ihren Nachkommen ist nicht effizient. Ein Verfahren, bei dem Chimären ausschließlich das Genom der injizierten ES-Zellen an ihre Nachkommen übertragen, wäre sehr willkommen.

Im Rahmen meiner Doktorarbeit habe ich zwei Studien durchgeführt, welche sich vorwiegend mit der Ableitung und Genregulation von XEN-Zelllinien beschäftigten. Dabei konnte ich zwei Erstautorenschaften erlangen. Außerdem konnte ich einen Beitrag zu einer Studie leisten in der die ausschließliche Übertragung des ES-Zell-Genoms in der Maus-Keimbahn gezeigt wurde. Bei dieser Arbeit fungierte ich als Zweitautor.

Efficient derivation of extraembryonic endoderm stem cell lines from mouse postimplantation embryos. Jiangwei Lin, Mona Khan, Bolek Zapiec, Peter Mombaerts. Scientific Reports, 2016

Im Jahre 2005 wurde erstmals die Gewinnung von XEN-Zellen aus Blastozysten beschrieben, seitdem gibt es zurzeit insgesamt drei Methoden um diese Zellen zu generieren. Wie zuvor genannt ist eine direkte Generierung aus Blastozysten möglich, aber durch die Umwandlung von ES-Zellen und die Reprogrammierung von Fibroblasten können ebenfalls XEN-Zellen gewonnen werden. Bisher ist noch unklar, ob eine Generierung dieser Zellen aus post implantierten Embryonen möglich ist.

Im Rahmen dieser Veröffentlichung habe ich eine schnelle und effiziente Methode etabliert, um XEN-Zellen aus Mausembryonen von Tag E5.5 – E 6.5 unter der Verwendung von disaggregierten als auch ganzen Embryonen zu gewinnen. Dabei konnte ich insgesamt 77 XEN-Zelllinien von 85 postimplantierten Embryonen im Alter von Tag E5.5 – E6.5 herstellen, parallel dazu war es mir möglich 41 XEN-Zelllinien von 69 präimplantierten Embryonen des Blastozystenstadiums zu erstellen. Eine 100 prozentige Erfolgsrate konnte unter der Verwendung von ganzen Embryonen an Tag E5.5 und disaggregierten Embryonen an Tag E6.5 erzielt werden. Immunfluoreszenz- und NanoString Genexpressionsanalysen weisen darauf hin, dass XEN-Zellen die aus postimplantierten Embryonen gewonnen wurden den XEN-Zellen, die ich im Rahmen dieser Arbeit aus präimplantierten Embryonen des Blastozystenstadiums (vor XEN) gewonnen habe, sehr ähnlich sind. Injiziert man diese gewonnenen Zellen in Blastozysten, tragen diese post XEN-Zellen dann zur Entwicklung des extraembryonalen Entoderms, in Chimären an Tag E6.5 und E7.5 insbesondere zu Entwicklung des parietalen Entoderms bei. Zusammenfassend konnte ich im Rahmen dieser Publikation eine hoch effiziente Methode für die Gewinnung von XEN-Zellen aus postimplantierten Embryonen, als auch von

disaggregierten Embryonen von Tag E 5.5 und E6.5 zeigen und das eine Gewinnung von XEN-Zellen aus präimplantierten Embryonen vergleichbare Zelllinien schafft.

PDGFRA is not essential for the derivation and maintenance of mouse extraembryonic endoderm stem cell lines. Jiangwei Lin, Mona Khan, Bolek Zapiec, Peter Mombaerts. Stem Cell Reports, 2017

Bisherigen Annahmen zur Folge ist für die Gewinnung sowie für die Erhaltung von XEN-Zellen der von Plättchen-abgeleitete-Wachstumsfaktor–Rezeptor A (PDGFRA) essentiell. Im Rahmen dieser Studie wurde diese These überprüft.

Durch die Verwendung PDGFRA defizienter XEN-Zelllinien, gewonnen aus prä- und postimplantierten Embryonen eines PDGFRA-GFP knockout Stammes, war es möglich die These des essentiellen Einflusses von PDGFRA zu überprüfen. Außerdem konnte aus PDGFRA defizienten ES-Zellen mit Hilfe der Verwendung von Retinsäure und Activin A, XEN-Zellen generiert werden. Das XEN Profil der insgesamt 12 verschiedenen Zelllinien wurde mit Hilfe der Immunfluoreszenz, der NanoString Genexpressionsanalyse und anhand ihres Einflusses auf das extraembryonale Entoderm von Chimären, welche durch die Injektion dieser Zellen in Blastozysten erzeugt wurden, untersucht. Anhand dieser Ergebnisse lässt sich daraus schließen, dass PDGFRA nicht essentiell für die Generierung und Kultivierung von XEN-Zellen ist.

Exclusive transmission of the embryonic stem cell-derived genome through the mouse germline. Frank Koentgen, **Jiangwei Lin**, Markella Katidou, Isabelle Chang, Mona Khan, Jacqui Watts, Peter Mombaerts. Genesis, 2016

Gene-Targeting in ES-Zellen ist nach wie vor die beste Methode, um komplexe Mutationen in der Keimbahn der Maus hervorzurufen. Ein wichtiger Aspekt in diesem mehrstufigen Prozess spielen die Logistik und ethische Vertretbarkeit der Zuchteffektivität bezogen auf die Keimbahnübertragung. Eine Übertragung des ES-Zell abgeleiteten Genoms von Chimären auf ihre Nachkommen spielt dabei die entscheidende Rolle. Es wurde eine Methode entwickelt bei der männliche Chimären ausschließlich das Genom der injizierten ES-Zellen an ihre Nachkommen übertragen. Diese neue Technologie, die als goGermline bezeichnet wird, beinhaltet die Injektion von ES-Zellen die aus der Verpaarung von homozygoten Tsc22d3-gefloxten Weibchen verpaart mit ROSA26-Cre Männchen,

gewonnen wurden. Aus dieser Kreuzung gehen Männchen hervor, die aufgrund eines zellautonomen Defektes in der Spermatogenese steril sind. Die resultierenden männlichen Chimären können steril sein, sind sie jedoch fruchtbar so übertragen sie das von ES-Zellen abgeleitete Genom zu 100 Prozent auf ihre Nachkommen. Diese Methode wurde umfassend in zwei verschiedenen Laboren auf genspezifische ES-Klone validiert, die von den üblicherweise verwendeten parenteralen ES-Zelllinien Bruce4, E14 und JM8A3 abgeleitet wurden. Die Geburt von unerwünschten nicht ES-Zell abgeleiteten Nachkommen in der goGermline Technologie unterbleibt und erfüllt die Anforderungen des 3R-Prinzips. Im Rahmen dieser Veröffentlichung habe ich die Mikroinjektion der Gen-gerichteten ES-Zellen in Blastozyten der goGermline Linie vorgenommen, um Chimären zu generieren. Die männlichen Chimären wurden dann mit Weibchen verpaart, um von den ES-Zellen abstammende Nachkommen zu produzieren.

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Part I

General introduction and discussion

CHAPTER 1

Introduction

1.1 Context

Research on the early development of mammalian embryos and embryo-derived stem cell lines is critical for progress in human reproductive biology and regenerative medicine. The mouse has been a productive model system for the human, for at four reasons. First, mouse blastocysts are morphologically similar to human blastocysts. Defects in the trophoblast and primitive endoderm (PrE) cell lineage can cause pregnancy loss, which affects 5% of couples trying to conceive (Stephenson and Kutteh, 2007). Second, embryonic stem (ES) cell lines can be derived from blastocysts, and contribute to all tissues including the germline upon injection into blastocysts (Evans and Kaufman, 1981; Martin, 1981; Bradley et al., 1984). Human embryonic stem cell (hESC) cell lines have great potential for applications in regenerative medicine, and gene targeting in ES cells has been used to generate thousands of mouse strains. Third, trophoblast stem (TS) cell lines have been derived from mouse blastocysts and from the extraembryonic ectoderm (ExE) of postimplantation embryos (Tanaka et al., 1998). TS cell lines reflect trophoblast cell development, contribute to the formation of the placenta, and are a useful model for studying embryo implantation into the uterus. Fourth, PrE cells contribute to the extraembryonic endoderm (ExEn) of the yolk sac, which protects the embryo and functions as an “early placenta,” providing nutrients for embryo development (Cross et al., 1994). This ExEn is required for inductive events such as anterior patterning and the formation of endothelial cells and blood islands (Byrd et al., 2002).

Extraembryonic endoderm stem (XEN) cells have been derived from PrE of blastocysts, converted from ES cells, and induced from fibroblast cells by OSKM (OCT4, SOX2, KLF4 and MYC) expression (Fujikura et al., 2002;

Kunath et al., 2005; Parenti et al., 2016). However, it was not known if XEN cells can also be derived from postimplantation embryos. XEN cells contribute to the gut endoderm in vivo (Kwon et al., 2008; Viotti et al., 2014), and serve as an alternate self-renewing source of extraembryonic endoderm-derived cells, which could be used to produce tissues for regenerative medicine (Niakan et al., 2013).

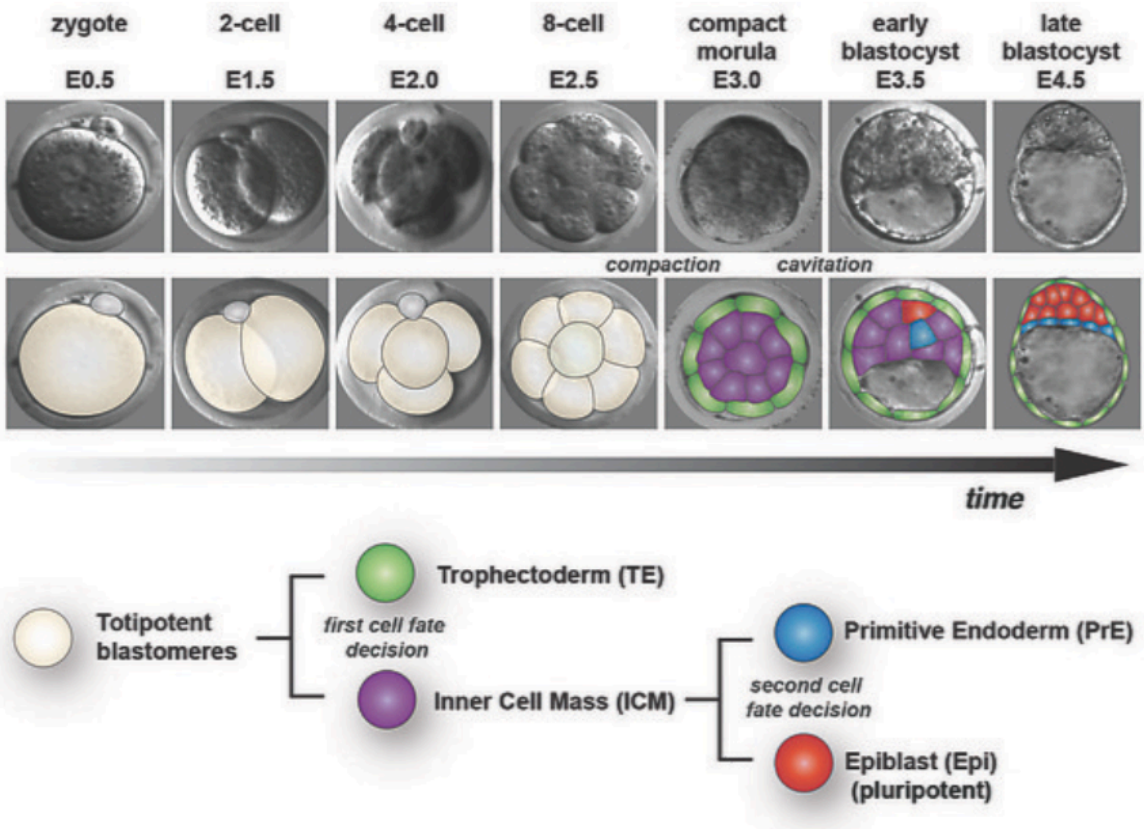
Gene targeting in ES cells remains the best practice for introducing complex mutations into the mouse germline. But the transmission of the ES cell-derived genome through the germline of chimeras to their offspring is not efficient. A method whereby chimeras transmit exclusively the genome of the injected ES cells to their offspring would be very welcome.

1.2 Background

1.2.1 Early embryo development

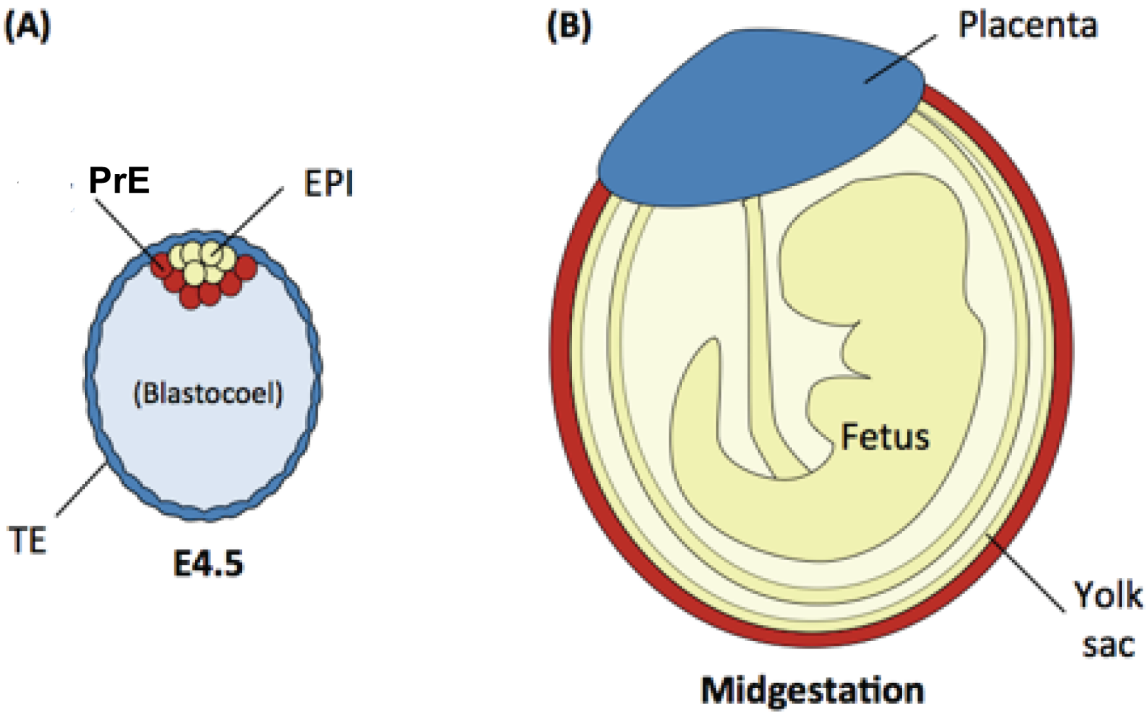
During mouse embryogenesis, the morula undergoes compaction and gradually differentiates into the trophectoderm (TE) around the outer cell mass and the inner cell mass (ICM) (Johnson and Ziomek, 1981). By E3.5, the second cell-fate decision takes place involving the segregation of ICM into the pluripotent epiblast (EPI) and PrE, which are distributed in a salt-and-pepper pattern. By the late blastocyst stage, PrE forms a layer of cells along the surface of the blastocoel cavity (Fig. 1). The epiblast gives rise to the embryo proper, amnion, and extraembryonic mesoderm of the yolk sac. TE cells give rise to the placenta. PrE forms the two ExEn lineages: visceral endoderm (VE) and parietal endoderm (PE) of the yolk sac (Chazaud et al., 2006; Plusa et al., 2008) (Fig. 2). VE surrounds the epiblast and ExE. PE cells migrate along the inner surface of TE and, together with trophoblast giant cells, secrete basement membrane proteins to form the Reichert's membrane (Hogan et al., 1980). The Reichert's membrane is composed of basement membrane proteins, including collagens and laminins, which lie between the PE and trophoblast cells. This membrane acts as a filter allowing the embryo

access to nutrients while forming a barrier to the maternal cells (Gardner, 1983).



(Niakan et al., 2013)

Figure 1. Cell-fate decision during early embryo development.
Blastomeres are totipotent from the zygote to the 8-cell stage, and the embryo makes the first cell-fate decision in the morula stage. The outer cell layer is the trophectoderm (TE) and the inner cell layer is the inner cell mass (ICM). After the second cell-fate decision, ICM differentiates into the PrE and epiblast.



(Frum et al., 2015)

Figure 2. Blastocyst lineages. (**A**) At E4.5, the blastocyst contains three cell types: epiblast (EPI), primitive endoderm (PrE) and trophectoderm (TE). (**B**) At midgestation, these three cell types correspond to fetus, yolk sac and placenta.

VE initially surrounds only the epiblast, and later covers ExE. By E5.0, VE cells surrounding the epiblast and ExE are morphologically and molecularly distinct, and they represent the embryonic VE (emVE) and extraembryonic VE (exVE). The emVE cells are flatter and more epithelial-like, while the exVE cells are columnar and cuboidal (Takito and Al-Awqati 2004). By E5.5, the emVE cells migrate proximally, initiating global VE cell migration (Takaoka et al., 2011). Shortly after emVE cell migration begins, a second VE population migrates into the proximal-anterior region of the embryo, forming the anterior VE (AVE) (Takaoka et al., 2011). The VE cells and their derivatives play a critical role in organization. The VE cells are the first site of hematopoiesis (Toles et al., 1989; McGrath and Pails 2005) and form the blood islands and endothelial cells through the expression of Indian hedgehog and vascular endothelial growth factor (VEGF) (Byrd et al., 2002; Damert et al., 2002). At

gastrulation, the emVE cells contribute to forming the gut endoderm tissue of the fetus (Kwon et al., 2008). VE and PE function as an “early placenta” that is responsible for nutrient and waste exchange (Cross et al., 1994). By E7.0, the anterior VE cells are dispersed along the boundary of the embryonic and extraembryonic regions and to the anterior half of the extraembryonic yolk sac (Stern et al., 2012). A transition to dependence on the “mature” placenta occurs at E10.0 (Cross et al., 1994).

1.2.2 Embryo-derived stem cells

Embryonic-derived stem (EDS) cells are derived from pre-implantation embryos and postimplantation embryos. EDS include three types of stem cell lines, which have been derived from the first three cell lineages of mouse embryos and have been used to study cell-fate decisions. ES cell lines can be derived from the epiblast of blastocysts and postimplantation embryos (Evans and Kaufman, 1981; Bao et al., 2009). Epiblast stem cell (EpiSC) are derived from preimplantation and postimplantation mouse embryos (Brons et al., 2007; Tesar et al., 2007; Najm et al., 2011). TS cell lines can be derived from the trophoblast of blastocysts and the postimplantation embryos, and also by the conversion of ES cells (Tanaka et al., 1998; Lu et al., 2008). XEN cell lines can be derived from blastocysts, by the conversion of ES cells, or by the induction of fibroblast cells by OSKM expression (Fujikura et al., 2002; Kunath et al., 2005; Parenti et al., 2016) (Fig. 3). Totipotent-like cells can convert from ES cells, which have the potential to differentiate into three cell lineages (Macfarlan et al., 2012). EDS cells are able to assemble into postimplantation embryos-like and blastocyst-like structures in vitro (Harrison et al., 2017; Kime et al., 2018; Rivron et al., 2018).

Sox17, Platelet-derived growth factor receptor alpha (PDGFRA) and Gata6 are required for XEN cell line derivation and maintenance (Artus et al., 2010; Niakan et al., 2010; Cho et al., 2012). XEN cell lines serve as a paradigm for XEN cell biology and the differentiation of the PrE into derivatives such as VE and PE. XEN cells show paternally imprinted X-chromosome inactivation and can serve as a model for understanding epigenetic modification (Kunath et al.,

2005). XEN cells contribute to forming the gut endoderm in vivo and serve as an alternate self-renewing source of extraembryonic endoderm-derived cells (Kwon et al., 2008; Viotti et al., 2014). XEN cells could be used to produce tissues for regenerative medicine (Niakan et al., 2013).

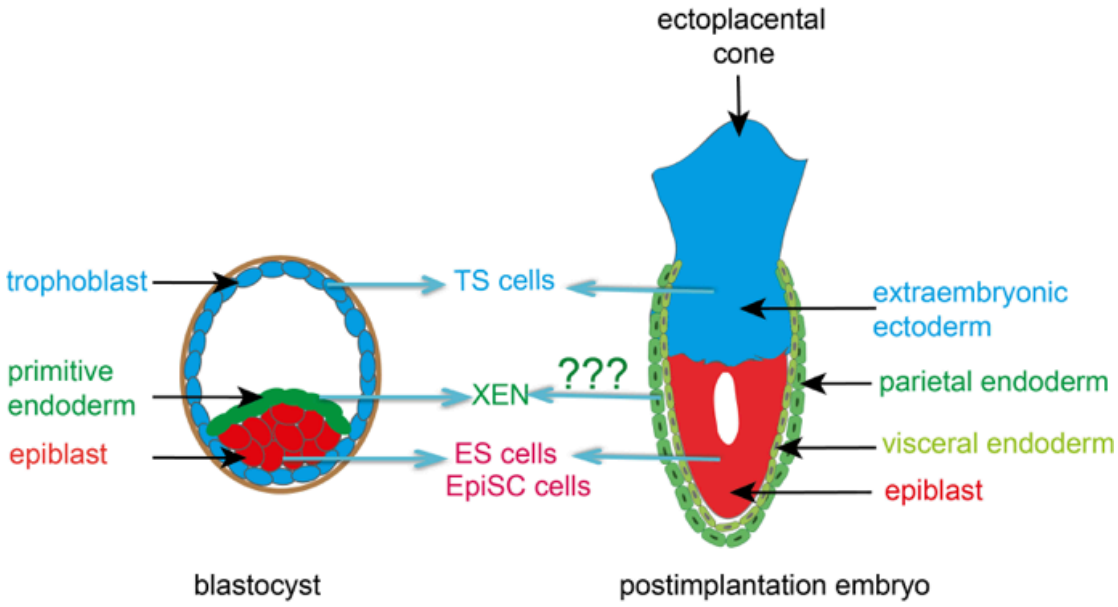


Figure 3. Embryonic-derived stem (EDS) cell lines from preimplantation and postimplantation embryos. After the second cell-fate decision, blastocysts have three lineages. ES cell and EpiSC are derived from the epiblast of blastocysts or postimplantation embryos. TS cell lines are derived from blastocysts or postimplantation embryos. XEN cell lines are derived from blastocysts.

1.2.2.1 Mouse ES cells

ES cells can be derived from blastocysts (Evans and Kaufman, 1981; Martin, 1981) and postimplantation embryos (Bao et al., 2009), and give rise to teratocarcinomas when grafted to adult mice and result in high-contribution chimeras and germ-line transmission to generate healthy offspring when injected into blastocysts (Bradley et al., 1984).

Through self-renewal, ES cells maintain pluripotency when cultured on mouse feeder cells in the presence of fetal bovine serum and leukemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988). In the absence of feeder cells, ES cells attach to gelatin-coated dishes and proliferate, but they also differentiate. Medium conditioned by preincubation with feeder cells cannot prevent differentiation but it does delay its onset. LIF can replace feeder cells for both the derivation and long-term culture of ES cells. LIF is produced by feeder cells (Rathjen et al., 1990). LIF is a principal factor, and feeder cells lacking the LIF gene barely support ES cells (Stewart et al., 1992). LIF maintains ES cell pluripotency by signaling through the LIF-JAK-STAT (Janus kinase/signal transducers and activators of transcription) pathway (Niwa et al., 1998). LIF-JAK-STAT signaling is mediated by Stat3, which regulates downstream pluripotency genes (Niwa et al., 2009). In the absence of serum, LIF alone is not sufficient for maintaining pluripotency but instead requires exogenous bone morphogenetic protein (BMP) signaling, a signaling pathway activated in the serum (Ying et al., 2003). Mouse ES cells can also be maintained in the absence of mouse embryonic fibroblast (MEF), serum, or BMP signaling when cultured in an inhibitor of FGFR2/Erk kinase (extracellular signal–regulated kinase) and GSK (glycogen synthase kinase) signaling (Ying et al., 2008).

ES cells express pluripotency transcription factors such as Oct4, Sox2, and Nanog, which promote ES cell self-renewal (Nichols et al., 1998; Chambers et al., 2003; Mitsui et al., 2003; Morrison and Brickman, 2006; Masui et al., 2007). ES cells express the cell-surface markers stage-specific embryonic antigen-1 (SSEA1) and platelet endothelial cell adhesion molecule-1 (PECAM1) (Canham et al., 2010; Rugg-Gunn et al., 2012). Oct4 is expressed in oocytes and at early embryo stages, and it is exclusively maintained in the germ cell lineage. Embryos fail to establish in Oct4 mutant mice, and ICMs differentiate into the trophoblast cell lineage (Nichols et al., 1998). ES cells fail to self-renew and they differentiate when Oct4 is deleted (Niwa et al., 2000). However, Oct4 overexpression does not enhance ES cell self-renewal but instead promotes ES cell differentiation (Niwa et al., 2000). Conversely, a reduced level of Oct4 has been found to affect ES cell differentiation without

affecting self-renewal (Karwacki-Neisius et al., 2013). Sox2-null mutations are lethal to early postimplantation embryos. Loss of Sox2 causes ES cells to differentiate into the trophoblast cell lineage, similar to that observed in Oct4 mutant mice, and Sox2 overexpression may reduce ES cell differentiation (Avilion et al., 2003; Masui et al., 2007). A Nanog knockout results in embryos that lose the epiblast and secondarily PrE. Conditional deletion of Nanog in ES cells makes them prone to differentiation, but they can still sustain ES cell self-renewal and contribute extensively to chimeras (Chambers et al., 2007). Although ES cells with a conditional deletion of Nanog contribute to primordial germ cells, they fail to mature in the genital ridge (Chambers et al., 2007), Esrrb (estrogen related receptor beta) complementation rescues development of Nanog-null germ cells (Zhang et al., 2018). ES cells with a conditional deletion of Blimp1 contribute to chimeras, but they fail to form germ cells in the genital ridge (Bao et al., 2012). Forced expression of Nanog sustains ES cell self-renewal without the requirement for LIF, BMP, or 2i (Chambers et al., 2003; Ying et al., 2003, 2008; Hall et al., 2009).

Naïve ES cells cultured with LIF and 2i are assumed to correspond to the epiblast of the blastocyst (Boroviak et al., 2014). However, there are several reports about heterogeneity in ES cells (Chambers et al., 2007; Dietrich and Hiiragi, 2007; Singh et al., 2007; Hayashi et al., 2008; Toyooka et al., 2008). By using Hex reporter ES cell lines, Canham et al., 2010 and Morgani et al., 2013 identified the precursor of ExEn in ES culture; Hex positive cells contribute to VE and PE after injection into blastocysts. In 2i culture conditions, a substantial proportion of cells with the Hex reporter could contribute to the TE. ES cells cultured in 2i or KSR (knockout serum replacement) resembled the cells of the ICM of E3.5 blastocysts in their transcriptome, and ES cells cultured in serum resembled the epiblast of E4.5 blastocysts. ES cells cultured in 2i or KSR could contribute to the TE in chimeras (Martin Gonzalez et al., 2016). So ES cells both have naïve-stage cells that correspond to epiblast of blastocyst and cells with the potential of the early ICM.

1.2.2.2 Epiblast stem cells

Epiblast stem cells (EpiSC) are derived from preimplantation and postimplantation mouse embryos and are different from mouse ES cells; moreover, they are more similar to human ES cells than mouse ES cells. EpiSC express the same core pluripotency transcription factors, such as Oct4, Nanog, and Sox2, as ES cells. However, Klf4, Rex1 (Zfp42), and Dppa3 are present in ES cells but absent in EpiSC (Brons et al., 2007; Tesar et al., 2007; Guo et al., 2009; Najm et al., 2011). Furthermore, Fgf5, Cer1, and Otx2 are present in EpiSC but absent in ES cells. EpiSC are dependent on FGF/Erk and Activin/Nodal signaling for self-renewal, which is similar to human ES cells, while ES cells also rely on JAK/STAT and BMP signaling for their self-renewal (Vallier et al., 2009). One epigenetic difference between ES cells and EpiSC is that female ES cells have two active X-chromosomes while female EpiSC have one already inactive X-chromosome. In addition, EpiSC do not survive well as isolated single cells and they need to be passaged in clumps. The characteristics of primed pluripotency in mouse EpiSC are shared with human hESC (Nichols et al., 2012). EpiSC can be derived from a wide range of developmental stages, spanning pre- and postimplantation development and differentiated from ES cells (Najm et al., 2011; Osorno and Chambers, 2011; Kojima et al., 2014). EpiSC differentiate into all three germ layers in teratoma assays (Brons et al., 2007; Tesar et al., 2007), but they do not effectively contribute to chimeras after injection into blastocysts (Huang et al., 2012). EpiSC still express pluripotency genes and can be reprogrammed to naïve pluripotency by transfection with only a single transcription factor, Klf4 (Guo et al., 2009), or by direct reversion to ES cells without transfection of any gene (Bao et al., 2009).

1.2.2.3 Trophoblast stem cells

TS cells can be generated from the polar TE of blastocysts or from the ExE of postimplantation embryos (up to E8.5), or by conversion from ES cells with the marker genes *Cdx2*, *Eomes*, and *Gata3*, which give rise to the mature trophoblast cells of the placenta (Tanaka et al., 1998; Lu et al., 2008; Chiu et

al., 2010; Golding et al., 2012; Hayakawa et al., 2015). In addition, induced TS cells can be derived from fibroblasts by transient expression of Tfap2c, Eomes, Gata3, and Ets2 (Kubaczka et al., 2015). TS cells cultured in the presence of FGF4 (fibroblast growth factor 4) /heparin and TGF-β/activin, or cultured in MEF-conditioned medium (CM), remain in an undifferentiated and self-renewing state (Tanaka et al., 1998; Erlebacher et al., 2004).

TS cells can also be derived and maintained in serum-free medium consisting of chemically defined ingredients on Matrigel, termed TX medium (Kubaczka et al., 2014). BMP4 induces mouse ES cells to the trophoblast cell lineage in defined culture condition on laminin (Hayashi et al., 2010). hESC express trophoblast marker after treatment with bone morphogenetic protein 4 (BMP4) in vitro (Xu et al., 2002). Combined with LIF, BMP4 also supports the self-renewal of mouse ES cells in defined culture conditions (Ying et al., 2003). LIF blocks ES cell conversion to TS cells, the LIF-STAT3 pathway blocks Lef1, and LIF inhibits CDX2 expression (He et al., 2008).

1.2.2.4 XEN cells

XEN cell lines are assumed to derive from the PrE lineage of blastocysts, which are cultured in the presence of FGF4 and heparin or ES medium with LIF (Kunath et al., 2005; Niakan et al., 2013). XEN cells share many characteristics with PE, but few with VE. XEN cells contribute mostly to PE and rarely to VE after being injected into blastocysts (Kunath et al., 2005; Lin et al., 2016). XEN cells are a heterogeneous population and contain two distinct morphologies, a small round highly refractile morphology and a stellate epithelial-like morphology (Kunath et al., 2005). XEN cells can differentiate into cells with a VE identity in vitro either by BMP signaling, by Nodal and Cripto signaling (Kruithof-de Julio et al., 2011; Artus et al., 2012; Paca et al., 2012), by laminin-mediated differentiation, or by high-density culture on gelatin-coated plates (Paca et al., 2012). When XEN cells differentiate into cells with a VE identity, they will have increased expression of AFP (alpha-fetoprotein), E-cadherin, Ihh, and Ttr (Paca et al., 2012). XEN cells differentiated into cells with a VE identity contribute to VE and AVE after

being injected into blastocysts (Kruithof-de Julio et al., 2011). XEN cells express PrE specific genes, such as *Gata6*, *Pdgfra*, *Sox17*, *Gata4*, *Sox7*, *Dab2*, and *Sparc* (Kunath et al., 2005). How do such genes regulate XEN cells? The Erk pathway is critical for XEN cell derivation, and Grb2 is a critical adaptor in the Grb2-Erk pathway. Grb2 mutations block PrE cell lineage in blastocysts and block the expression of PrE-specific genes, such as *Gata6*, *Pdgfra*, *Sox17*, *Gata4*, and *Sox7* (Chazaud et al., 2006). Fgfr2 is critical for the Grb2-Erk pathway, and inhibition of Fgfr2 by PD173074 or inhibition of MEK by PD0325901 has similar effects as Grb2 mutations (Nichols et al., 2009; Yamanaka et al., 2010). The active Erk pathway induces first Gata6 expression and then the other PrE specific genes. Gata6 mutations completely block the subsequent expression of genes, such as *Pdgfra*, *Sox17*, *Gata4*, and *Sox7* (Bessonnard et al., 2014; Schrode et al., 2014). Because Fgf4 can be replaced by FGF2, Fgf4 is not essential for the establishment of XEN cell lines; however, Fgfr2 could be essential for the establishment of XEN cell lines (Kang et al., 2013). Sall4 is essential for derivation of XEN cell lines. Sall4 seems to play a role as an activator of key lineage-defining genes in the ExEn (Lim et al. 2008). ExEn is hypomethylated when compared with embryonic tissue (Chapman et al., 1984; Monk et al., 1987; Gardner & Davies,1992). XEN cells express low levels of H3K27me3 (Rugg-Gunn et al., 2010). Thus, hypomethylation of ExEn could enable the tissue to easily undergo differentiation or transdifferentiation.

PDGFRA and Sox17 are important to form the PrE cell lineage. Deletion of PDGFRA or Sox17 decreases the number of PrE cells (Artus et al., 2011, 2013). PDGFRA-mutant embryos can still develop to term but with severe defects, and they die soon after birth (Ogura et al., 1998). Sox17 mutant embryos cannot survive past the E8.0 stage (Artus et al., 2013). There are reports that PDGFRA and Sox17 are essential for deriving XEN cell lines (Artus et al., 2010; Niakan et al., 2010; Cho et al., 2012). We hypothesized that XEN cell lines can be derived from PDGFRA-mutant embryos and ES cells, because the remaining PrE still has the ability to support embryo development. It is still not clear which genes regulate XEN cell derivation and maintenance.

Human XEN cell lines have not been derived from human blastocysts. This failure could be due to differences in the growth factors that support XEN cell progenitors between humans and mice (van Kuijk et al., 2012; Roode et al., 2012). Although overexpression of endodermal transcription factors results in expression of many endoderm markers, it is unclear what the molecular characteristics of XEN cells are (Séguin et al., 2008; Wamaitha et al., 2015). A rat extra-embryonic endoderm precursor (XEN-P) cell line has been derived from rat blastocysts; the XEN-P cell line expresses Oct4 and SSEA1 at high levels, its growth is stimulated by LIF, and cells express Gata6 and Gata4. XEN-P cells can contribute to ExEn after injection into rat blastocysts (Debeb et al., 2009). Zhong et al., 2018 reported the isolation of mouse primitive extraembryonic endoderm stem cell (pXEN) lines from mouse blastocysts, which express Oct4 and share characteristics with rat XEN-P cells. pXEN cells are highly similar to XEN cells by morphology, gene expression profile and lineage contribution. pXEN cells can convert into XEN-like cells, but not vice versa. pXEN cells are more representative than XEN cells of PrE of the blastocyst stage (Zhong et al., 2018). However, in this report, it is unclear if pXEN cells can contribute efficiently to VE-like PrE. In another recent report, mouse ES cells converted to XEN-like cells termed naïve extraembryonic endodermal (nEnd) cells, with characteristics close to blastocyst-stage ExEn precursors, by adding Activin A, LIF and Chir99021 in the culture medium (Anderson et al., 2017). Interestingly, rodent multipotent adult progenitor cell (MAPC) derived from bone marrow in rat multipotent adult progenitor cell (rMAPC) medium (Lo Nigro et al., 2012) express Oct4 and Rex1 but not Nanog and Sox2. However, MAPC express Gata4, Gata6, Sox7, Sox17, which are expressed in the PrE (Nichols et al., 2011) and in rat XEN-P cells (Debeb et al., 2009). Rat XEN-P cell lines derived from rat blastocysts in rMAPC medium, which resemble E3.5 nascent hypoblasts, were termed rat hypoblast stem cells (rHypoSC) (Lo Nigro et al., 2012). When green fluorescent protein (GFP)-labeled rMAPC and rHyoSCs were aggregated with rat morulae, both types of cells contribute to ExEn (Lo Nigro et al., 2012). Since bone marrow-derived MAPC have similar characteristics to rHypoSCs, some bone marrow cells may originate from PrE. It is unclear that XEN cells

can convert into bone marrow cells.

Mouse fibroblasts pass via a XEN-like state on their way to induced pluripotent stem cell (iPSC) by chemical reprogramming (Zhao et al., 2015). The same group reported that chemically induced pluripotent stem cells pass via a XEN-like stage to a 2C-like stage (early embryonic-like) to become iPS cells (Zhao et al., 2018). In the early or middle blastocyst stage, epiblast precursors can convert to PrE precursors spontaneously, and conversely, PrE precursors can convert to epiblast precursors as well (Grabarek et al., 2012). However, in the E4.5 blastocyst, epiblast precursors show less plasticity than precursors of PrE, probably owing to differences in responsiveness to extracellular signaling (Grabarek et al., 2012). Using single-cell resolution quantitative imaging, Xenopoulos et al., 2015 noted an irreversible commitment to epiblast/PrE lineages in vivo and showed that rare cells from PrE can convert into epiblast, but not vice versa. In vitro, ES cells can convert into XEN cells spontaneously (Lin et al., 2017; Lo Nigro et al., 2017). However, there is no report that XEN cells can convert into ES cells spontaneously. XEN cells could be converted into pluripotent stem cells by chemical induction (Zhao et al., 2015). It could be that XEN-like cells induced by chemicals are more similar to PrE-like cells and that the PrE-like cells convert to pluripotent stem cells.

1.2.2.5 Totipotent cells

The zygote and 2-cell embryos are totipotent, in that they are able to develop into all embryo tissues and extraembryonic tissues such as placenta and extraembryonic membranes encasing the embryo (Tarkowski et al., 1959; Papaioannou et al., 1989). ES cell lines are derived from the ICM of blastocysts and are thought to be equivalent to the ICM, which lacks the ability to contribute to extraembryonic tissue (Evans and Kaufman, 1981; Martin et al., 1981). ES cells contribute rarely to extraembryonic tissues (Beddington et al., 1989), and these cell cultures could be contaminated by trophoblast and PrE derived cells. A two-cell (2C) stage embryo-specifically expressed repetitive element, called murine endogenous retrovirus with

leucine tRNA primer (MuERV-L), is expressed in a low percentage of ES cells in culture (approx. 0.5%); the cells lack Oct4, Nanog and Sox2 expression, but have the ability to contribute to epiblast lineages and extraembryonic lineages after injection into morulae. These cells are called 2C-like cells (Macfarlan et al., 2012).

Expanded potential stem cell (EPSC) have been established from eight-cell blastomeres using a cocktail of inhibitors and LIF as expanded potential stem cell medium (EPSCM). These EPSCs have the ability to contribute to ICM and trophoblast after injection the cells into morulae and then contribute to E14.5 chimeras in the embryo proper and in the extra-embryonic tissues. Also, conventional ES cells after five passages in EPSCM acquired the potential to contribute to the trophoblast (Yang et al., 2017). EPSC displayed high expression of genes similar to 4-cell to 8-cell stage blastomeres. TS cell lines and XEN cell lines can be derived from EPSC (Yang et al., 2017). But there is no report that TS or XEN cell lines can be isolated from 2C-like cells (Macfarlan et al., 2012). 2C-like cells convert from ES cells spontaneously or after treatment with TSA (Macfarlan et al., 2012). 2C-like cells can be induced through downregulation of the chromatin assembly activity of CAF-1, and 2C-like cells exhibit higher reprogrammability than ES cells upon nuclear transfer (Ishiuchi et al., 2015). DUX4, a eutherian-specific multicopy retrogene, activates hundreds of endogenous genes and the MERVL family that defines the cleavage-specific transcriptional programs in mice. Mouse DUX is necessary and sufficient to induce mouse ES cells into 2C-like cells (Hendrickson et al., 2017), but stable 2C-like cell lines have not been reported.

Totipotent cells have also been observed in naïve ES cells cultured in the 2i (two inhibitor: PD0325901 and Chir99021) condition. A rare Hex (ExEn marker) positive cell appeared in the cultured embryos and ES cells, after adding 2i. Single Hex positive ES cells coexpressed epiblast and extraembryonic genes and contribute to all lineages in chimeras (Morgani et al., 2013).

1.2.2.6 Embryos assembled from embryo-derived stem cells

From blastocysts, it is possible to derive TS, XEN, and ES cell lines (Tanaka et al., 1998; Evans and Kaufman, 1981; Kunath et al., 2005), which in vitro resemble the trophoblast, primitive endoderm and epiblast respectively. Can embryo-derived stem cells assemble into embryos in vitro spontaneously? Several papers reported that postimplantation embryo-like and blastocyst-like structures could be obtained by assembling ES cells with TS cells, or EpiSC in 3D (three-dimensional) culture or in microwells (Harrison et al., 2017; Rivron et al., 2018; Kime et al., 2018). Harrison et al., 2017 reported that combining mouse ES cells and TS cells in a three-dimensional scaffold generated structures whose morphogenesis is similar to postimplantation embryos (Harrison et al., 2017). However, ES and TS cells derived embryos lack ExEn. It may be possible to obtain postimplantation embryo-like structures by combining ES cells and TS cells with XEN cells. Recently human postimplantation amniotic sac embryoid (PASE) that recapitulate multiple postimplantation embryogenic events centered around amniotic sac development, were assembled by human pluripotent stem cells (Shao et al., 2017). TS cells combined with ES cells in vitro form structures that morphologically and transcriptionally resemble E3.5 blastocysts (Rivron et al., 2018) and EpiSC produces blastocysts-like hemispheres while the EpiSC convert into naïve pluripotent cells (Kime et al., 2018).

1.2.3 PrE and XEN cell genes

The model of sequential marker activation (Gata6 > PDGFRA > Sox17 > Gata4 > Sox7) within cells of the PrE lineage (Artus et al., 2010) is consistent with the failure of Gata6 mutant embryos to activate the sequential expression of *Pdgfra*, *Sox17*, and *Gata4* in PrE of blastocysts (Schrode et al., 2014). Gata6 mutants exhibit a complete absence of the PrE, while Sox17 or PDGFRA mutants exhibit only a reduced number of PrE cells (Artus et al., 2011, 2013; Schrode et al., 2014). This means that Sox17 or PDGFRA mutants could be partially rescued by other genes or pathways. Grb2 mutants have no expression of Gata6, PDGFRA, Sox17, Gata4, and Sox7, and no PrE

formation in blastocysts (Chazaud et al., 2006); therefore, Grb2 is required for Erk pathway and PrE and XEN cell lineage. GATA6 is downstream from Grb2 in the inductive signaling pathway and the expression of GATA6 is sufficient to compensate for the defects caused by Grb2 deficiency in the development of the primitive and extraembryonic endoderm (Wang et al., 2011). The absence of upstream genes could lead to lack of expression of downstream genes.

1.2.3.1 Gata6

Gata6 is the first gene to be expressed in the PrE, which is first detected in the 8-cell stage blastomere, and later co-expressed with *Nanog* in the ICMs of early blastocysts stage (Chazaud et al., 2006; Plusa et al., 2008). During embryo development, individual ICM cells exclusively express Gata6 or Nanog for the specification of the PrE and the epiblast (Chazaud et al., 2006; Plusa et al., 2008). Gata6 mutant embryos fail to form PrE, all the ICMs express Oct4 and Sox2 and fail to activate other PrE marker genes such as *Pdgfra*, *Sox17*, *Gata4,* and *Sox7* (Schrode et al., 2014). This suggests that all PrE cells prematurely commit to the epiblast fate, similar to what is observed in Grb2 mutant embryos and when the Fgfr2 is blocked by PD173074. In vitro, Gata6 is essential for XEN cell self-renewal (Lim et al., 2008). Ectopic expression of Gata6 is sufficient to promote ES cells to convert to XEN cell lines (Fujikura et al., 2002; Shimosato et al., 2007; Artus et al., 2010). In Gata6 heterozygous embryos, the number of PrE cells is reduced and their commitment is decelerated (Schrode et al., 2014). The Gata6 mutation affects VE development in the embryo but does not produce ExEn defects until after the formation of blastocysts (Morrisey et al., 1998; Koutsourakis et al., 1999).

1.2.3.2 *Pdgfra*

Platelet-derived growth factor receptor alpha (PDGFRA) is a tyrosine kinase receptor. Platelet-derived growth factors (PDGFs) have been implicated in the control of cell proliferation, survival, and migration. A PDGFRA knokcout results in embryonic lethality between E8 and E16. PDGFRA-null embryos

display a complex phenotype characterized by a cleft face, abnormally patterned somites, subepidermal blebbing, spina bifida, cervical vertebrae and rib fusions, malformation of the shoulder girdle, and hemorrhaging (Soriano et al., 1997; Tallquist et al., 2000; Klinghoffer et al., 2002). Furthermore, PDGFRA signaling is required for normal development of many cell types, including oligodendrocytes, lung alveolar myofibroblasts, intestinal mesenchyme, and Leydig cells (Betsholtz et al., 2001). PDGFRA expression coincides with expression of GATA6, the earliest expressed transcriptional regulator of the PrE lineage. PDGFRA is initially expressed at the 16–32 cell stages, is then found in the PrE of blastocysts, and after implantation is expressed in both VE and PE of E5.5–E7.5 embryos, and in XEN cell lines (Artus et al., 2010). PDGFRA is a good marker to identify PrE cell lineages and XEN cell lines. PDGFRA is required for PrE cell survival in the ICM of mouse blastocysts (Artus et al., 2013). PDGFRA-mutant blastocysts have significantly decreased numbers of PrE cells. PDGFRA is also assumed to be essential for the derivation and maintain of XEN cell lines. Gata6 is required for PDGFRA expression by ES cells during their conversion into XEN cells upon retinoic acid (RA) treatment (Artus et al., 2010).

1.2.3.3 *Sox17*

Sox17 is a member of the Sry-related high-mobility group box (Sox) transcription factors and has an essential role in the differentiation of several types of cells (Foster et al., 1994; Schilham et al., 1996; Kamachi et al., 1998; Pingault et al. 1998). During mouse embryogenesis, Sox17 is first detected at the 16–32 cell stages co-expressed with Oct4, then in PrE of blastocysts, and later in VE at E6.0 and in the endoderm at E7.5, where it plays an essential role in organ formation (Kanai-Azuma et al., 2002). Previous studies have revealed its role in the regulation of fetal hematopoiesis (Kim et al., 2007) and vasculogenesis (Matsui et al., 2006; Sakamoto et al., 2007). Sox17 also has been proposed to function as a key regulator of endoderm formation and differentiation, a function that is conserved across vertebrates (Hudson et al., 1997; Alexander et al., 1999; Clements et al., 2000). In the mouse, genetic inactivation of Sox17 leads to severe defects in the formation of the definitive

endoderm (Kanai-Azuma et al., 2002). Sox17 is critical for PrE formation, and lack of Sox17 significantly decreases the PrE numbers of blastocysts (Artus et al., 2011). XEN cell lines cannot be derived from Sox17 mutant embryos and converted from ES cells (Niakan et al., 2010; Cho et al.2012). Downregulation of Sox17 by RNA interference impairs XEN cell maintenance (Lim et al., 2008). Embryonic bodies derived from Sox17 mutant ES cells fail to correctly form the outer ExEn layer (Niakan et al., 2010). Sox17 mutant ES cells differentiate to PrE but fail to differentiate to PE and VE fates (Shimoda et al., 2007). Overexpression of Sox17 is sufficient to promote ES cells to convert to XEN cells (McDonald et al., 2014).

1.2.3.4 *Gata4*

GATA4 is essential for the early development of multiple organs, including the heart, foregut, liver, and ventral pancreas (Kuo et al., 1997; Molkentin et al., 1997; Watt et al., 2004, 2007). Interestingly, Gata4 is also expressed in the genital ridge and is required for formation of the genital ridge (Hu et al., 2013). During early embryo development, Gata4 is detected at the 64-cell stage, after Gata6, PDGFRA, and Sox17 activation, and is only detected in Gata6-positive ICM cells (Plusa et al., 2008). Mutations of Gata6 block Gata4 expression in the PrE and XEN cells. However, the mutations of the earlier expressed genes such as PDGFRA or Sox17 cannot block Gata4 expression. Gata4 mutant ES cells differentiate to ExEn cells upon RA treatment, and like wild-type cells, ExEn cells express PDGFRA, Gata6, Sox17, Sox7, and Foxa2 (Artus et al., 2010), indicating that Gata4 is not essential for differentiation into ExEn cells. It is unclear how Gata6 regulates Gata4 expression.

1.2.3.5 *Sox7*

Sox7 is expressed in PrE in the late blastocyst and in PE and VE in postimplantation embryos (Artus et al., 2012). Sox7-null embryos have a lethal phenotype before E14.5, with heart development failure (Wat et al., 2012). Sox7 is dispensable for PrE differentiation from mouse ES cells (Kinoshita et al., 2015). Sox7 overexpression in mouse ES cells does not

drive the cells to PrE cell fate completely, or it has little effect on ExEn gene expression (Futaki et al., 2004; Kinoshita et al., 2015). However, Sox7 overexpression in human ES cells activates ExEn genes (Séguin et al., 2008).

1.2.4 XEN cell signaling pathways and gene regulation

1.2.4.1 The Erk signaling pathway

Robust Erk pathway activity (downstream from Fgf4) is essential for multilineage differentiation of ES cells (Kunath et al., 2007; Ying et al., 2008); therefore, inhibiting it promotes self-renewal. Fgf is the major stimulus activating Erk in mouse ES cells (Kunath et al., 2007). The Fgf-Fgfr2-MEK1/2-Erk signaling pathway is the critical pathway to segregate the epiblast and the PrE in pre-implantation embryos. Besides the main Fgf-Erk pathway, there are the PDGFRA-Erk (Chen et al., 2011) and LIF-Erk pathways (Niwa et al., 2009). FGF4, Fgfr2, and Grb2 are essential for PrE specification in mouse blastocysts (Lanner et al., 2014). Additionally, FGF4 in cultured blastomeres promotes nearly all ICMs to become PrE cells (Yamanaka et al., 2010). Fgf4 is required for lineage restriction and the salt-and-pepper distribution of the PrE, although Fgf4 is not essential for the derivation of XEN cell lines and it can be replaced by Fgf2 (Kang et al., 2013). Fgf4 is the product of epiblast cells or ES cells; however, since Oct4-null mutants block Fgf4 expression, it has been suggested that Fgf4 is a target of Oct4 (Yuan et al., 1995; Nichols et al., 1998; Frum et al., 2013). Fgfr2 is expressed on the surface of nascent PrE cells, which bind to Fgf4, activate the Erk pathway, inhibit Nanog, and promote Gata6 expression to activate downstream PrE genes (Lanner et al., 2014). In XEN cell lines, inhibitors of MEK activity slow XEN cell proliferation (Artus et al., 2010) and lead to upregulation of markers of VE (Spruce et al., 2010).

1.2.4.2 The Nodal and BMP signaling pathways

XEN cells do not contribute well to VE (Kunath et al., 2005). BMP4 and Nodal, members of the transforming growth factor (TGF)β superfamily, can induce

XEN cells to VE in vitro and in vivo (Kruithof-de Julio et al., 2011; Artus et al., 2012; Paca et al., 2012). The Nodal and Cripto signaling pathway differentiates XEN cells into VE/AVE, and the BMP signaling pathway induces XEN cells to differentiate into the extraembryonic VE (Kruithof-de Julio et al., 2011; Artus et al., 2012; Paca et al., 2012). Nodal signaling through Alk4 depends on Cripto and results in Smad2 phosphorylation. This canonical pathway then leads to VE and AVE differentiation. In the absence of Nodal, Cripto signals through Alk4 to promote Smad2 phosphorylation, which may lead to VE and AVE differentiation. This activity could be mediated through Alk4 binding by an unknown TGF-β ligand distinct from Nodal. However, the ability of Cripto to promote VE and AVE differentiation is partially independent of Alk4 kinase activity and Smad2 phosphorylation (Kruithof-de Julio et al., 2011).

1.2.4.3 Gene regulation in XEN cells

Prdm14 is a PR-domain and zinc-finger protein that is expressed in ES cells. Prdm14 safeguards mouse ES maintenance by preventing induction of the ExEn fate. Overexpression of Prdm14 prevents ES cells differentiation into ExEn cells, and knockdown or depletion of Prdm14 leads to the expression of ExEn genes (Ma et al., 2001). Activin/Nodal and wingless-type MMTV integration site family (Wnt) signaling induce ES cells to differentiate into the PrE cell lineage and also support ES cells self-renewal. LIF also induces ES cells to differentiate into the PrE cell lineage and supports ES cells self-renewal. Insulin fine-tunes ES cells self-renewal, and removal of insulin induces ES cells to differentiate into naïve XEN cells (Anderson et al., 2017).

CHAPTER 2

Summary and discussion

2.1 Summary

To investigate the derivation and gene regulation of XEN cell lines, I have conducted two studies, and published two papers as first author. In addition, I contributed to a paper as second author, wherein we developed a new approach of achieving exclusive transmission of the ES cell-derived genome through the mouse germline.

Efficient derivation of extraembryonic endoderm stem cell lines from mouse postimplantation embryos.
Jiangwei Lin, Mona Khan, Bolek Zapiec, Peter Mombaerts.
Scientific Reports, 2016

Since the first report of XEN cell derivation from blastocysts in 2005, there are three types of methods in place to derive XEN cell lines, either directly from blastocysts, by conversion from ES cells, or by reprogramming from fibroblasts. It is unclear if XEN cell lines can also be derived from postimplantation embryos.

Here, I have developed a fast and efficient method to derive mouse XEN cell lines from E5.5–E6.5 postimplantation embryos using disaggregated embryos or whole embryos. I derived 77 XEN cell lines from 85 postimplantation embryos at E5.5 or E6.5, in parallel to 41 XEN lines from 69 preimplantation embryos at the blastocyst stage. A success rate of 100% of XEN cell line derivation was achieved with E5.5 whole-embryo and E6.5 disaggregated-embryo methods. Immunofluorescence and NanoString gene expression analyses indicate that the XEN cell lines that I derived from postimplantation embryos (post-XEN) are very similar to the XEN cell lines that I derived from preimplantation embryos (pre-XEN) using a conventional method. After

injection into blastocysts, post-XEN cells contribute to ExEn, particularly to the PE in chimeras at E6.5 and E7.5. I show that XEN cell lines can be derived with high efficiency from postimplantation embryos at E5.5 or E6.5, either from whole or disaggregated embryos. These post-XEN cell lines are similar to the pre-XEN cell lines that were derived directly from preimplantation embryos.

PDGFRA is not essential for the derivation and maintenance of mouse extraembryonic endoderm stem cell lines.
Jiangwei Lin, Mona Khan, Bolek Zapiec, Peter Mombaerts.
Stem Cell Reports, 2017

PDGFRA is thought to be essential for the derivation and maintenance of mouse XEN cell lines. Here, I have re-evaluated the requirement for PDGFRA in the derivation and maintenance of XEN cell lines.

I derived multiple PDGFRA-deficient XEN cell lines from postimplantation and preimplantation embryos of a PDGFRA-GFP knockout strain. I also converted PDGFRA-deficient ES cell lines into XEN cell lines chemically by transient culturing with retinoic acid and Activin A. I confirmed the XEN profile of these 12 PDGFRA-deficient cell lines by immunofluorescence with various markers, by nanoString gene expression analyses, and by their contribution to the ExEn of chimeric embryos produced by injecting these cells into blastocysts. I conclude that PDGFRA is not essential for the derivation and maintenance of XEN cell lines.

Exclusive transmission of the embryonic stem cell-derived genome through the mouse germline.
Frank Koentgen, **Jiangwei Lin**, Markella Katidou, Isabelle Chang, Mona Khan, Jacqui Watts, Peter Mombaerts.
Genesis, 2016

Gene targeting in ES cells remains best practice for introducing complex mutations into the mouse germline. One aspect in this multistep process that

has not been streamlined with regard to the logistics and ethics of mouse breeding is the efficiency of germline transmission: the transmission of the ES cell-derived genome through the germline of chimeras to their offspring. A method whereby male chimeras transmit exclusively the genome of the injected ES cells to their offspring has been developed. The new technology, which is referred to as goGermline, entails injecting ES cells into blastocysts produced by superovulated homozygous Tsc22d3 floxed females mated with homozygous ROSA26-Cre males. This cross produces males that are sterile due to a complete cell-autonomous defect in spermatogenesis. The resulting male chimeras can be sterile but when fertile, they transmit the ES cell-derived genome to 100% of their offspring. The method was validated extensively and in two laboratories for gene-targeted ES clones that were derived from the commonly used parental ES cell lines Bruce4, E14, and JM8A3. The complete elimination of the collateral birth of undesired, non-ES cell-derived offspring in goGermline technology fulfills the reduction imperative of the 3R principle of humane experimental technique with animals. In this paper, I performed microinjection of gene-targeted ES cells into blastocysts to generate chimeras. The male chimeras were mated with females to produce ES cell-derived offspring.

2.2 Discussion

I have derived post-XEN cell lines from postimplantation embryos with high efficiency (Lin et al., 2016). I have also derived PDGFRA-deficient XEN cell lines from blastocysts and postimplantation embryos, and converted XEN cell lines from ES cell lines (Lin et al., 2017). I have contributed to the development of goGermline, a new technology that enables transmission of the ES cell-derived genome to 100% of the offspring of male chimeras (Koentgen et al., 2016).

Derivation of post-XEN cell lines from postimplantation embryos

I derived post-XEN cell lines from E5.5 embryos and disaggregated E6.5 embryos with a success rate of 100% (Lin et al., 2016). But the success rate of XEN cell line derivation from blastocysts is only 56% in ES medium with LIF and 21% in TS medium with F4H (25 ng/ml FGF4 and 1 mg/ml heparin) (Niakan et al., 2013). Why is the derivation of post-XEN cell lines more efficient than that of pre-XEN cell lines? There are many more ExEn cells in an E5.5 embryo (~95 ExEn cells) and in an E6.5 embryo (~250 ExEn cells) than in a blastocyst (~11 PrE cells) (Morris et al., 2010; Snow et al., 1977). Another reason could be that post-XEN cell lines are not only derived from ExEn cells but also from epiblast of postimplantation embryos, and that epiblast of postimplantation embryos is much easier to convert into XEN cells than epiblast of blastocysts.

XEN cell lines could be derived directly from PrE and ExEn

It is still unclear whether pre-XEN and post-XEN cell lines are derived directly from PrE and ExEn. If Pre-XEN cell lines are derived from PrE, it could be that PrE cells differentiate into XEN cells. PrE cells express some genes that are not expressed in pre-XEN cells, such as Amn (Ohnishi et al., 2014). Pre-XEN cells are similar to PE in terms of gene expression, pre-XEN cells injected into blastocysts contribute mostly to PE, and rare cells contribute to VE (Kunath et al., 2005). It remains to be determined if naïve XEN cells (or primitive XEN cells) can be derived in culture. Recently, mouse p-XEN cell lines were derived from blastocysts that express Oct4 and XEN-specific genes, but it is

unclear if these p-XEN cells can contribute efficiently to VE and PE, especially to VE (Zhong et al., 2018). ExEn cells are differentiated cells, which originate from PrE, and if they become post-XEN cells, the process could be via reprogramming by culture medium. Induction of reprogramming by culture medium has been reported for spermatogonial stem cells (Guan et al., 2006; Kanatsu-Shinohara et al., 2008; Ko et al., 2009) and for EpiSC (Bao et al., 2009) that de-differentiate into ES cell-like cells when cultured under ES cell conditions. It is still unclear if post-XEN cells originate from PE or/and VE. VE cells trans-differentiate to PE cells and PE cells mimic characteristics of XEN cells (Hogan et al., 1981; Ninomiya et al., 2005). Thus, it is possible that post-XEN cells also originate from PE and VE.

XEN cell lines could be derived indirectly from epiblast of preimplantation and postimplantation embryos

It is possible that pre-XEN and post-XEN cells originate not only from PrE and ExEn but also from epiblast of preimplantation and postimplantation embryos. The phenotype of ES cell heterogeneity has been reported by several labs (Chambers et al., 2007; Dietrich and Hiiragi, 2007; Singh et al., 2007; Hayashi et al., 2008; Toyooka et al., 2008). ES cells retain the ability to give rise to XEN-like cells spontaneously from the minor population expressing PDGFRA or Hex (Morgani et al. 2013; Lo Nigro et al., 2017). It would, therefore, be not surprising that conversion of epiblast-derived cells into XEN cells may occur during the derivation process from mouse pre- and postimplantation embryos. However, the establishment of XEN cells from postimplantation embryos is a novel and interesting phenomenon. ES cell lines can be derived from earlier postimplantation embryos (Bao et al., 2009), and EpiSC cannot convert into XEN cell line (Cho et al., 2012), suggesting that this conversion could happen via an ES-like intermediate state other than epiblast stem-like intermediate state.

In the process of deriving pre-XEN cell lines from blastocysts, the success rate in ES medium with LIF (56%) is higher than in TS cell medium with F4H (21%) (Niakan et al., 2013). ES medium with LIF is typically used to derive ES cell lines and in parallel XEN cell lines, and TS cell medium with F4H to derive

TS cell lines and in parallel XEN cell lines (Kang et al., 2013). The reason that the derivation of pre-XEN cell lines in ES medium with LIF is higher than in TS medium with F4H, could be that LIF promotes epiblast (ES-like) cells growth, and then enough epiblast (ES-like) cells convert into XEN cells. LIF promotes PrE cells in blastocysts (Morgani et al., 2015) and it could also promote XEN cells expansion. LIF, Activin and Wnt signaling not only induce PrE-like differentiation from ES cells but also support PrE-like expansion (Anderson et al., 2017). Thus, it could be that pre-XEN cells derived in ES medium with LIF have more cells originating from epiblast, which improves the success rate of pre-XEN cell lines.

Whole embryos could be faster to convert into post-XEN cells than disaggregated embryos

I have derived post-XEN cell lines from postimplantation embryos by the whole-embryo method or the disaggregated-embryo method. The disaggregated-embryo method has a 100% successful rate in E6.5 embryos, but the whole-embryo method is faster in producing post-XEN cell lines. The reason could be that the whole embryo is easier to reprogram or to convert into post-XEN cell line. I did not observe flat colonies (epiblast-like) in the culture by the whole-embryo method after passaging the outgrowths. But such flat colonies (epiblast-like) were always in the culture by the disaggregated-embryo method in the first few days. These epiblast-like cells could not keep flat colonies after passaging by disaggregation to single cells with TryPLE Express and culture in TS medium with F4H. I observed that a lot of single cells died after passaging with TryPLE Express, similar to the report that EpiSC undergo widespread cell death by passaging using trypsin or other single-cell dissociation methods (Brons et al., 2007).

ES cells in crowded culture conditions tend to differentiate. The epiblast of postimplantation embryos could have the same phenotype, explaining perhaps why the whole-embryo method is faster at producing post-XEN cell lines than the disaggregated-embryo method. Why did I obtain 100% post-XEN cell lines with the disaggregated-embryo method, and not with the whole-embryo method? One explanation is that the ectoplacental cone and

EXE would inhibit XEN cell growth. In the whole-embryo method, I removed the ectoplacental cone as much as possible, but there is still some tissue left. In the disaggregated-embryo method, I totally remove the ectoplacental core and EXE. The trophoblast-derived cells could also affect pre-XEN derivation. There are many trophoblast-derived cells surrounding the outgrowths during derivation of pre-XEN cell lines from blastocysts. I had to disaggregate the outgrowths on day 5 to day 7, in order to remove the trophoblast-derived cells that could affect XEN cell growth. However, for the outgrowths from isolated ICMs, in which the trophoblast is excluded, it is not necessary to disaggregate the outgrowth on day 5 to day 7. It is unclear if ES medium with LIF would support the derivation of post-XEN cell lines from postimplantation embryos. As XEN-like cells can arise from fibroblasts induced by OSKM and cultured in ES medium with LIF (Parenti et al., 2016), it could be that post-XEN cells arise in ES medium with LIF from postimplantation embryos.

OCT4 and Nanog are expressed in the epiblast of E6.5 embryos (Acampora et al., 2013). In the disaggregated-embryo method, epiblast cells formed flat colonies with OCT4 and NANOG expression (data not shown), and then the flat colonies gradually converted into XEN cells that surround the flat colonies. In the whole-embryo method, epiblast of E6.5 embryos mostly lose OCT4 and NANOG expression (data not shown), they do not form the flat colonies and directly convert into XEN cells. Thus, I speculate that XEN cell lines originate both from PrE and ExEn, and from epiblast of pre-implantation or postimplantation embryos.

The possible process of conversion of epiblast cells into XEN cell in of preimplantation and postimplantation embryos

How can epiblast of blastocysts and postimplantation embryos contribute to XEN cell lines? ICM comprises three distinct cell types. The first type coexpresses NANOG and GATA6, and differentiates to epiblast or to PrE; the second type forms epiblast; and the third type forms PrE (Frum et al., 2015). ES cells could go from an epiblast-like stage to a NANOG+ GATA6+ coexpression stage, over to a PrE-like stage, and then convert into XEN cells (Schröter et al., 2015). I speculate that, likewise, cells from a blastocyst could

go from the NANOG+ GATA6- epiblast stage to the NANOG+ GATA6+ coexpression stage, over to a NANOG- GATA6+ PrE stage, and then contribute to a pre-XEN cell line. Similarly, epiblast of postimplantation embryos could pass through a NANOG+ GATA6+ stage to a NANOG- GATA6+ stage, and contribute to a post-XEN cell line. Epiblast cells of postimplantation embryo differentiate from epiblast of the blastocyst and could be much easier to convert into XEN cells than ES cells.

TS, EpiSC, and ES cell lines were not derived in the cultures

During the derivation of pre-XEN cell lines in ES medium with LIF, typically ES cell lines are obtained together with pre-XEN cell lines, and when TS medium with F4H is used, typically TS cell lines are obtained together with pre-XEN cell lines (Kang et al., 2013). However, during the derivation of post-XEN cell lines, I did not obtain TS cell lines, EpiSC lines or ES cell lines from postimplantation embryos in TS medium with F4H (Lin et al., 2016). By immunofluorescence, cell lines derived from postimplantation embryos did not express the trophoblast marker Cdx2, and by NanoString analyses, cells did not express TS specific genes either. Why are TS cell lines not obtained from TS medium by my method? The protocol for deriving TS cell lines from postimplantation embryos involves discarding the VE and PE, and only keeping the ExE for culture (Hayakawa et al., 2015). I speculate that VE and PE cells inhibit TS cells growth and that VE and PE-derived cells grow much faster than TS cells and would outcompete TS cells with time. Another reason could be that the C57BL/6J background is not suitable for TS cell derivation (Hayakawa et al., 2015). In my experiments, I used C57BL/6J or C57BL/6J crossed with CD1. TS cell lines could be derived successfully from CD1 and 129 mice (Hayakawa et al., 2015). The culture method could affect the derivation of TS cell lines. For XEN cell line derivation, the larger the outgrowth, the better it is to derive XEN cell lines. But for TS cell line derivation, the large outgrowths affect TS derivation, possibly by inducing differentiation of TS cells. Although ES cells can be derived from postimplantation embryos, the cells need to be cultured in ES medium with LIF (Bao et al., 2009), it is impossible to derive ES cells in TS medium with F4H. EpiSC derivation and maintenance requires Activin A and FGF2 (Brons

et al., 2007), so it is difficult to derive EpiSC in the TS medium without Activin A and FGF2.

The possible reasons for the establishment of PDGFRA-deficient XEN cell lines

I have also derived PDGFRA-deficient XEN cell lines from blastocysts and postimplantation embryos, and converted XEN cell lines from ES cell lines (Lin et al., 2017). Why can PDGFRA-deficient XEN cell lines be converted from ES cells by infrequent cell passaging and the method of collecting the floating cells? Why are PDGFRA-deficient pre-XEN cells difficult to derive from preimplantation embryos? Why are PDGFRA-deficient post-XEN cells easy to derive from postimplantation embryos? For the PDGFRA-deficient ES cells conversion to XEN cells, first, I used the infrequent cell passaging method to induce differentiation of PDGFRA-deficient ES cells, by changing the medium frequently and without passaging the cells often. Crowded ES cells could have difficulty maintaining their pluripotency, and then gradually differentiate. I believe that infrequent passaging pushes the ES cells to differentiate. I found that the XEN-like cells tend to be in suspension. The reason could be that in crowded culture conditions, the XEN-like cells were excluded by ES cells or other differentiating cells. The conventional method involves frequent passaging of cells, and the ES cells do not exit the pluripotent stage. PDGFRA-deficient ES cells are more difficult to differentiate than PDGFRA-heterozygous ES cells in TS medium with F4H. Second, I collected the floating cells. I collected the suspended cells in the medium and spun them down to accumulate GFP+ cells (XEN-like) in a new dish. I found that XEN cells cultured in TS medium are more in suspension than when cultured ES medium with LIF while cells colonies become crowded in TS or ES medium. The conventional method to change medium and passaging cells involves the removal of the medium, which removes the suspended (XEN-like) cells. I obtained four PDGFRA-deficient XEN cell lines from the infrequent cell passaging method and the method of collecting the floating cells. Third, a low dose of RA (0.01 μM) and Activin A (10 ng/ml) promotes ES cell conversion into XEN-like cells (Cho et al., 2012). But in my experiments, a high concentration of RA (1 μM) would induce XEN-like cells to die, especially in

PDGFRA-deficient ES cells. Embryos lacking PDGFRA signaling exhibit increased cell death within the PrE lineage, and a pan-caspase inhibitor (Z-VAD) (Talanian et al., 1997) can rescue the number of PrE cells in PDGFR mutant embryos (Artus et al., 2013).

Thus, combining the RA and Activin A treatment, with the infrequent cell passaging method, and with the collection of floating cells, it is the best method to converted PDGFRA deficient ES cells into XEN cell lines. For the derivation of XEN cell lines from PDGFRA-deficient blastocysts, the missing PDGFRA signal reduces the number of PrE (Artus et al., 2013). But the remaining PrE still has the ability to support fetus development to the prenatal stage (Hamilton et al., 2003), and I obtained six PDGFRA-deficient fetuses from a total of 28 fetuses (20%). As ES cells grow much faster than XEN cells, frequent cell passaging would dilute XEN cells. I noticed that GFP+ cells (PDGFRA-deficient XEN-like cells) are still kept in the cultures and that they grow slowly and form large colonies. ES cells formed huge colonies, and then they grow slower than the ES cells, which were disaggregated to single cells. However, after passaging the XEN cell colonies and ES cell colonies by TryPLE Express into single cells, it is difficult to find XEN cells (GFP+ cells) in the cultures. The reason is that the ES cells grow much faster than XEN cells and then become dominant after several passages. So, it is necessary to pick the XEN-like cells colonies during pre-XEN derivation from blastocysts. For the derivation of post-XEN cell lines , it is easier to derive PDGFRA-deficient XEN cell lines from postimplantation embryos (E6.5) than from blastocysts, because the pluripotent epiblast has already differentiated and cells could convert into XEN cells spontaneously. If there are any epiblast stem-like cells and TS cells in the derivation process, they may be inhibited by XEN cells. XEN cells grow faster than TS cells, and epiblast stem-like cells would die after passaging into single cells with TryPLE Express (Brons et al., 2007), therefore XEN cells could be dominant in the culture with time. Post-XEN cell lines could be derived from ExEn and converted from epiblast of postimplantation embryos. It could be that postimplantation embryos have more ExEn cells than PrE for derivation (Morris et al., 2010; Snow et al., 1977).

Endogenous FGF4 is sufficient to convert PDGFRA deficient ES cells into XEN cells

Endogenous FGF4 is sufficient to convert ES cells into XEN cells (Cho et al., 2012), which could be also sufficient to convert PDGFRA-deficient ES cells into XEN-like cells in my culture conditions. By performing immunofluorescence, I found PDGFRA-GFP and Sox17 expression in ES-23 (PDGFRA-deficient) and ES-18 (PDGFRA-heterozygous) cultured in TS medium for 30 days with or without F4H. However, Oct4 was still expressed in the cells, but it is unclear if GFP+ cells and Sox17+ cells co-localize with Oct4+ cells.

FGF4 is produced first by the epiblast in blastocysts, and also produced by ES cells. Oct4-deficient blastocysts cannot produce FGF4, they express the earliest PrE-gene Gata6, but no PrE late gene Sox17 (Le Bin et al., 2014). However, additional FGF4 can induce Sox17 expression in Oct4-deficient blastocyst (Le Bin et al., 2014).

Deficiency of PDGFRA could be rescued by endogenous FGF

Why is PDGFRA not essential for the derivation of XEN cells? PDGFRA activates the Erk pathway (Chen et al., 2011), which is similar to the FGFR2-Erk pathway. FGF4 deficiency would inhibit XEN cell derivation from blastocysts, and the endogenous PDGF signaling pathway cannot rescue the Erk pathway. FGF4 activation of the Erk pathway would be rescued by the addition of FGF2 since FGF2 binds Fgfr1 to activate the Erk pathway (Kang et al., 2013). The Erk pathway of PDGFRA-deficient cells could be rescued by endogenous FGF. It is possible that sustained MEK/Erk activity is required for the specification and maintenance of the PrE lineage, and that its activity could be initially regulated by FGF signaling, and then reinforced by PDGFR signaling (Artus et al., 2012).

PDGFRA deficiency may inhibit ES cells exiting from pluripotency

Inhibition of FGFR is critical for inhibiting ES cells exit pluripotency (Ying et al., 2008); however, PDGFRA could be still important for ES cells to exit

pluripotency. In my experiments, PDGFRA-deficient cells can maintain the ES cell phenotype in TS cell medium without LIF, even with the addition of F4H. I cultured ES-23 in TS medium with F4H for 9 days and then injected ES cells into 46 C57BL/6J blastocysts. I obtained 34 E13.5 fetuses, of which 12 were chimeras with GFP expression. Next, I cultured ES-23 in TS medium with F4H for 21 days and then injected them into goGermline blastocysts (Koentgen et al., 2016). I obtained 8 pups by natural birth from 21 injected blastocysts. Five of these were chimeras; unfortunately, four of 5 chimeras with a high contribution from ES cells died before weaning, and only one chimera with minor ES cell contribution survived to adulthood. Similarly, PDGFRA-deficient pups die after birth (Hamilton et al., 2003). FGFR and PDGFRA could switch on and turn off the Erk pathway to regulate ES cell pluripotency and differentiation.

PDGFRA-deficient XEN cells could be rescued by Sox17

When the Erk pathway is activated, the earliest expressing gene in the PrE or XEN cells is Gata6, then PDGFRA and Sox17 are expressed, later Gata4 is expressed, and the latest gene expressed is Sox7 (Kang et al., 2013). I speculate that PDGFRA and Sox17 have parallel expression because Sox17 or PDGFRA-deficient cells in blastocysts cannot block each other's expression (Artus et al., 2013; Artus et al., 2011). By contrast, Gata6 mutant cells will block all the subsequent XEN cell genes from being expressed (Schrode et al., 2014). I speculate that PDGFRA-deficient XEN cells could be rescued by Sox17 in parallel expression, and conversely, that Sox17 mutant cells could be rescued by PDGFRA in the PrE and in XEN cell derivation and maintenance in the parallel expression.

Complete germline transmission of the ES-cell derived genome from male chimeras by the goGermline technology

I have demonstrated that the goGermline technology can be applied to obtain 100% germline transmission of the ES-cell-derived genome from male chimeras (Koentgen et al., 2016). Homozygous Tsc22d3 floxed females were mated with homozygous ROSA26-Cre males to obtain F1 blastocysts as host blastocysts for ES cell injection. Males carrying Tsc22d3 knockout alleles

were reported to be sterile (Bruscoli et al., 2012; Romero et al., 2012; Suarez et al., 2012; Ngo et al., 2013); this sterility is due to a cell-autonomous defect in spermatogenesis. The testes of hemizygous Tsc22d3 knockout males supported normal spermatogenesis when transplanted with wild-type germ cells (Bruscoli et al., 2012), suggesting that these testes would also support ES cell-derived germ cells to develop into normal sperm.

Although recent advances in gene editing methods, e.g., CRISPR/Cas9 technology, could achieve efficient gene knockout or knockin mouse zygote and thereby obviates the intermediary vehicle of ES cells (Wang et al., 2013; Yang et al., 2013, 2014), mosaicism in gene editing and off-target effects remain problematic for direct gene editing of mouse embryos. But gene targeting in ES cells for complex genetic modifications and knockins of large deoxyribonucleic acid (DNA) segments remains best practice. The rate of germline transmission after injection of cells into tetraploid blastocysts is also 100%, but the birth rate is low (Nagy et al., 1993), and it is not possible to obtain homozygous mice if the mutation is lethal. However, with ES cells carrying a homozygous mutation in a gene that is essential for survival, I could still get chimeras and germline transmission, after injection into diploid blastocysts. But when diploid blastocysts are used as hosts for ES injection, host-derived pups are obtained in parallel to ES-derived pups, and sometimes the ES-derived are is rare (Bradley et al., 1984). To obtain complete germline transmission from the injected ES cells, a sterile host would be needed. The Perfect Host approach (Taft et al., 2013) promised to improve germline transmission rates by generating male chimeras with diphtheria-toxin mediated ablation of the host germline. But this approach, as it was described, is imperfect in that it disregards the practical advantages of coat color differences between strains used to derive the ES cell lines and strains used to produce blastocysts. Moreover, the Perfect Host approach was tested only for 11 gene-targeted ES cell clones and in only one laboratory. A major advantage over the Perfect Host approach (Taft et al., 2013) is that the goGermline technology exploits fully the practical advantages of coat color differences for identifying chimeras and ES cell-derived offspring.

2.3 Conclusions and prospects

Conclusions

I have derived post-XEN cell lines from postimplantation embryos with high efficiency (Lin et al., 2016). I found that PDGFRA is not essential for derivation and maintenance of XEN cell lines (Lin et al., 2017). I contributed to developing a perfect method to obtain germline transmission from goGermline blastocysts (Koentgen et al., 2016).

Prospects

For XEN cell biology, there are a lot of questions that remain unanswered. First, the origin of pre-XEN cell lines remains unclear. Because pre-XEN cells share genes expression with PrE, they are assumed to originate from PrE (Kunath et al., 2005). But as ES cells and epiblast of blastocyst can convert to XEN-like cells or PrE (Grabarek et al., 2012; Lo Nigro et al., 2017), XEN cell lines may originate not only from PrE but also from the epiblast. Second, the origin of post-XEN cell lines remains unclear. It could be that some post-XEN cells originate from epiblast of postimplantation embryos. It is unclear whether post-XEN cell lines are also from ExEn (PE and/or VE). It is also unclear how the epiblast and ExEn to convert into post-XEN cell lines. Third, the signaling pathway and gene regulation in XEN cells are not well understood. It was reported that PDGFRA is required for derivation of XEN cell lines (Artus et al., 2010; Cho et al., 2012; Artus et al., 2013), and that Sox17, Gata6, Gata4 are also required for deviation of XEN cell lines (Niakan et al., 2010; Cho et al., 2012). Here, I reported that PDGFRA-deficient XEN cell lines can be derived from blastocysts and postimplantation embryos, and can be converted from ES cells. In Sox17-deficient blastocysts, the PrE cell number is significantly reduced, but they still have some PrE cells (Artus et al., 2011), similar to what is observed in PDGFRA-deficient blastocysts. I speculate that PDGFRA-deficient XEN cells could be rescued by Sox17 in parallel expression and that Sox17 mutant cells could be rescued by PDGFRA. So, Sox17-deficient ES cells could also convert into XEN cell lines. Fourth, the Erk signaling pathway

is essential for derivation of XEN cell lines. It is unclear that, when the Erk signaling pathway is blocked by the inhibitor PD0325901, another signaling pathway can rescue it so that XEN cell lines can be derived. Fifth, induction of pluripotent stem cells by chemical reprogramming occurs via a XEN-like stage (Zhao et al., 2015). It remains unclear how XEN cells convert into pluripotent stem cells. ES cell can convert into XEN cells spontaneously (Lo Nigro et al., 2017; Niakan et al., 2010), but XEN cells cannot convert into ES cells. Sixth, nEnd and pXEN cell lines have been isolated from ES cells and blastocyst (Anderson et al., 2017; Zhong et al., 2018). nEnd and pXEN express the PrE specific genes *Oct4*, *Zfp42/Rex1*, *Nr0b1/Dax1*, which are not expressed or lowly expressed in conventional XEN cells. The extent of similarity of nEnd and pXEN are PrE cells could be determined by single-cell RNA-sequencing. It is unclear if nEnd and pXEN can effectively contribute to VE. Seventh, it is unclear if naïve XEN cells or PrE could improve the development of cloned embryos after replacing the PrE from cloned embryos with naïve XEN cells or PrE from fertilization-derived embryos. Previously I found that the trophoblast cell lineage is the main defect to impair the development of cloned embryos to term (Lin et al., 2011). I separated the ICMs from cloned blastocysts and aggregated the cloned ICM with two fertilization-derived tetraploid embryos. I found that the full-term development of cloned ICMs was dramatically improved after the trophoblast cells in the cloned blastocysts were replaced by cells from tetraploid embryos, thus providing direct evidence that defects in trophoblast cell lineage underlie the low success rate of somatic nuclear transfer (Lin et al., 2011). However, the birth rate from the cloned aggregated embryos, in which trophoblast was replaced by fertilization-derived tetraploid embryos, was only half of the fertilization-derived blastocysts. I speculate that the cloned PrE may have defects, which could be rescued by fertilization-derived PrE or naïve XEN cells.

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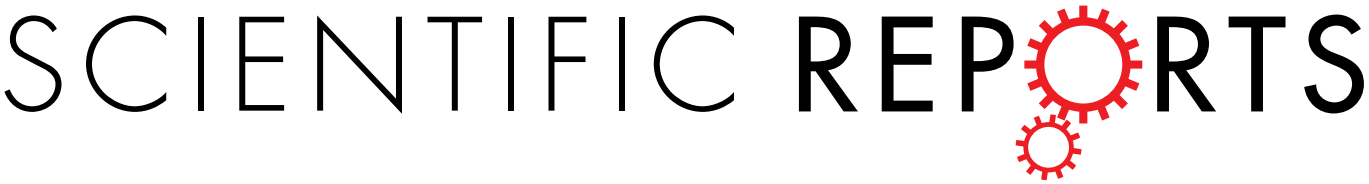
Part II

Efficient derivation of extraembryonic endoderm stem cell lines from mouse postimplantation embryos



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Efficient derivation of extraembryonic endoderm stem cell lines from mouse postimplantation embryos

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Various types of stem cell lines have been derived from preimplantation or postimplantation mouse embryos: embryonic stem cell lines, epiblast stem cell lines, and trophoblast stem cell lines. It is not known if extraembryonic endoderm stem (XEN) cell lines can be derived from postimplantation mouse embryos. Here, we report the derivation of 77 XEN cell lines from 85 postimplantation embryos at embryonic day E5.5 or E6.5, in parallel to the derivation of 41 XEN lines from 69 preimplantation embryos at the blastocyst stage. We attain a success rate of 100% of XEN cell line derivation with our E5.5 whole-embryo and E6.5 disaggregated-embryo methods. Immunofluorescence and NanoString gene expression analyses indicate that the XEN cell lines that we derived from postimplantation embryos (post-XEN) are very similar to the XEN cell lines that we derived from preimplantation embryos (pre-XEN) using a conventional method. After injection into blastocysts, post-XEN cells contribute to extraembryonic endoderm in chimeras at E6.5 and E7.5.

Mouse preimplantation embryonic development culminates in the blastocyst stage. A blastocyst consists of three cell lineages: epiblast, trophectoderm, and primitive endoderm (PrE). The epiblast develops into most of the embryo proper, the amnion, and the extraembryonic mesoderm of the yolk sac; the trophectoderm gives rise ultimately to the fetal portion of the placenta; and the primitive endoderm forms the two extraembryonic endoderm lineages – the visceral endoderm (VE) and the parietal endoderm (PE) of the yolk sac[1,2]. The extraembryonic endoderm provides nutritive support to the embryo, and is required for several inductive events such as anterior patterning and formation of endothelial cells and blood islands[3–5].

Stem cell lines have been derived from these three cell lineages[6]. Embryonic stem (ES) cell lines from epiblast were first reported in the 1980 s (refs 7 and 8), trophoblast stem (TS) cell lines from trophectoderm in the 1990 s (ref. 9), and extraembryonic endoderm stem (XEN) cell lines from PrE in the 2000 s (ref. 10). The conventional source of these cell lines is the blastocyst stage embryo. TS cell lines can also be derived from postimplantation embryos[9,11,12]. Moreover, mouse epiblast stem cell (EpiSC) lines, which resemble ES cell lines of human, can be derived from preimplantation embryos[13] and postimplantation embryos[14,15], and can be reverted to ES cells[16]. XEN cell lines are useful for the investigation of signaling pathways of cells of the extraembryonic endoderm lineages, and represent an *in vitro* model to identify patterning activities of the extraembryonic endoderm such as factors involved in cardiac induction[17,18]. Mouse fibroblasts pass via a XEN-like state on their way to induced pluripotent stem (iPS) cells by chemical reprogramming[19].

There are three methods to derive mouse XEN cell lines[20]. The first method entails the direct derivation of XEN cell lines from blastocysts[10]. The second method involves the conversion of an existing ES cell line to a XEN or XEN-like cell line, either by forced expression of a transcription factor gene encoding *Gata4* or *Gata6* (refs 21–23) or *Sox17* (refs 24 and 25), or by chemical modification of the culture medium such as by addition of retinoic acid and activin A[26]. A third, more recently reported method, derives induced XEN cells (iXEN) by reprogramming fibroblasts with the classical iPS reprogramming factors *Oct4*, *Sox2*, *Klf4*, and *Myc*; colonies from which iXEN cells can be derived, arise in parallel to iPS cells[27].

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Method	Stage of embryo for derivation	Strain	No. embryos	No. XEN cell lines (%)	No. XEN cell lines heterozygous	No. XEN cell lines wild-type
Whole embryo	Blastocysts	B6D2F1 × D4/XEGFP	20	11 (55)	11	0
Whole embryo	Blastocysts	D4/XEGFP × DBA/2 N	20	11 (55)	11	0
Whole embryo	Blastocysts	PDGFRa-GFP× CAG::mRFP1	23	14 (61)	14	0
ICMs	Blastocysts	R26-tauGFP41 × Sox17-Cre	6	5 (83)	2	3
		Sum	69	41 (59)		
Whole embryo	E6.5	Xist1loxGFP ×DBA/2 N	19	15 (79)	5	10
Whole embryo	E6.5	Gata6-mTomato × Cdx2-GFP	6	6 (100)	3	3
Whole embryo	E6.5	ROSA-STOP-taulacZ × Sox17-Cre	13	9 (70)	1	8
		Sum	38	30 (79)		
Disaggregated embryo	E6.5	R26-tauGFP41 × Sox17-Cre	11	11 (100)	5	6
Disaggregated embryo	E6.5	CD1 × PDGFRa-GFP	12	12 (100)	5	7
Disaggregated embryo	E6.5	ROSA-STOP-taulacZ × Sox17-Cre	7	7 (100)	1	6
		Sum	30	30 (100)		
Whole embryo	E5.5	R26-tauGFP41 × Sox17-Cre	13	13 (100)	11	2
Whole embryo	E5.5	ROSA-STOP-taulacZ × Sox17-Cre	4	4 (100)	2	2
	E5.5	Sum	17	17 (100)		

Table 1. Derivation of pre-XEN and post-XEN cell lines.

Here, we show that XEN cell lines can be derived with very high efficiency from postimplantation embryos at E5.5 or E6.5, either from whole or disaggregated embryos. These so-called post-XEN cell lines are very similar to the pre-XEN cell lines that we derived directly from preimplantation embryos.

Results

Derivation of pre-XEN cell lines from blastocysts. To distinguish unambiguously the XEN cell lines that were derived from preimplantation embryos from the XEN cell lines that were derived from postimplantation embyros, we refer to these cell lines operationally as pre-XEN and post-XEN cell lines, respectively.

We first derived a set of conventional pre-XEN cell lines from blastocysts, in order to set up conditions in our laboratory and to provide a comparison for post-XEN cell lines. We collected 63 E1.5 embryos from three types of natural matings: from three heterozygous PDGFRa-GFP females[28] mated with homozygous CAG::mRFP1 males[29], two B6D2F1 females mated with hemizygous D4/XEGFP males[30], and two homozygous D4/XEGFP females mated with DBA/2 N males (Table 1). PDGFRa is a XEN-cell marker[26,31,32]; CAG::mRFP1 is a transgene that expresses ubiquitously the red fluorescent protein; D4/XEGFP is a transgene integrated on the X-chromosome, and is not expressed from the inactive X-chromosome, enabling the future study of X-chromosome inactivation in XEN cell lines. We cultured the embryos in KSOM medium until the blastocyst stage, and then removed the zona pellucida using acid Tyrode solution. We transferred each blastocyst separately into a well of a 4 well-dish coated with 0.1% gelatin and covered with mouse embryonic fibroblasts (MEF), and switched to ES cell medium supplemented with leukemia inhibitory factor (LIF)[20]. All blastocyst cells including PrE cells express RFP from the transgene (Fig. 1a). After 3 days in culture, blastocysts started to form outgrowths (Fig. 1a), which we disaggregated on day 5. Cell line X47 was still in the process of becoming established (and was still displaying intrinsic red fluorescence) after 19 days in culture (Fig. 1a). We obtained a stable pre-XEN cell line X47 after 38 days of culture (Fig. 1a). The CAG promoter does not appear to generate sufficient mRFP1 to enable us to detect the intrinsic red fluorescence in XEN cell lines, similar to what has been reported for CAG::H2B-GFP and CAG::YFP strains[26]. We thus derived, using the conventional method[20] with ES cell medium and LIF, a total of 36 pre-XEN cell lines from 63 blastocysts, at a 57% success rate (Table 1).

Next, we isolated by immunosurgery[33] the inner cell mass (ICM) of blastocysts from a natural mating between an R26-tauGFP41 female and a Sox17-Cre male[34]. With Sox17 a XEN-cell marker, and R26-tauGFP41 a Cre reporter strain, this cross marks permanently cells that have been derived from Sox17-expressing cells. We obtained 6 ICMs from 6 blastocysts, which we had collected at E2.5 as morulae and cultured overnight in KSOM medium. We transferred each ICM separately into a well of a 4-well dish coated with gelatin and covered with MEF, and cultured the ICM outgrowths in ES medium with LIF. Seven days later we replaced the ES medium by TS medium with 25 ng/ml FGF4 and 1 μg/ml heparin (referred to as F4H) without passaging the cells. We changed the medium every two days, and on day 14 we passaged the cells to 12-well dishes. We thus established five XEN cell lines from six ICMs, at a success rate of 83% (Table 1).

Figure 1b shows immunofluorescence staining of pre-XEN cell line X42, from the cross PDGFRa-GFP × CAG::mRFP1. Cells also display intrinsic green fluorescence of GFP produced from the gene-targeted *PDGFRa-GFP* locus (indicated with the asterisk PDGFRa-GFP*). We find that this and other pre-XEN cell lines are immunoreactive for XEN cell markers GATA4, GATA6, SOX7, SOX17, and DAB2, but negative for ES cell markers OCT4 and NANOG, and negative for TS cell marker CDX2.

Derivation of post-XEN cell lines from whole E6.5 embryos. Next we collected E6.5 postimplantation embryos from three types of natural matings: two heterozygous Xist1loxGFP females[35] mated with a wild-type DBA/2 N male, two heterozygous ROSA26-STOP-taulacZ females mated with a heterozygous

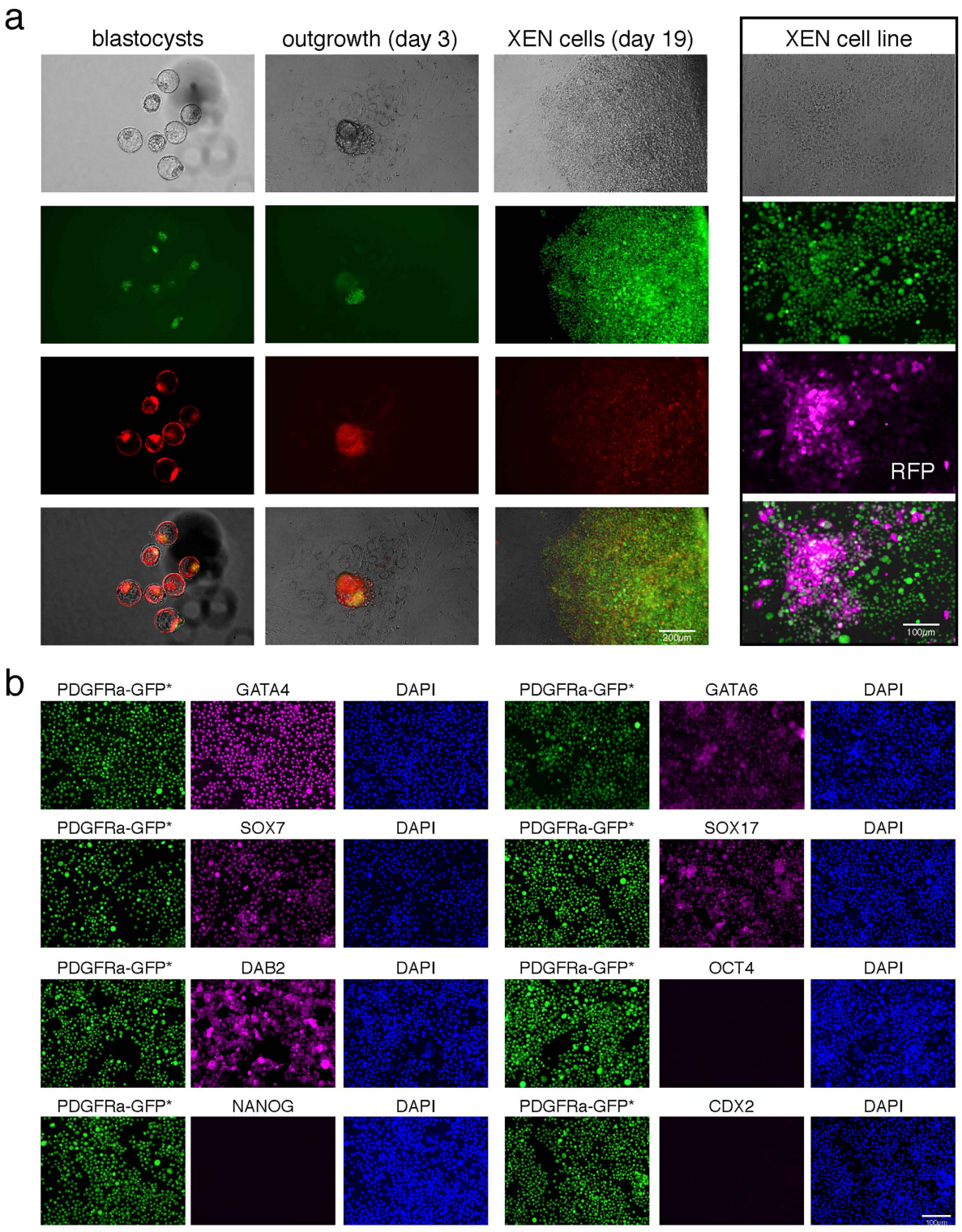

Figure 1. Derivation of pre-XEN cell lines from blastocysts. (**a**) PDGFRa-GFP × CAG::mRFP1 blastocysts and pre-XEN cell line X47. From left to right: blastocysts, outgrowth of blastocyst after 3 days in culture, cells after disaggregation of the outgrowth and culture for 19 days, and established pre-XEN cell line on day 38. From top to bottom, first three columns: bright-field image, intrinsic green fluorescence of GFP, intrinsic red fluorescence of RFP, and combined green and red fluorescence/bright-field image. From top to bottom, right-most column: bright-field image, intrinsic green fluorescence of GFP, immunofluorescence for RFP, and combined green fluorescence and immunofluorescence/bright-field image. (**b**) Fluorescence analysis of pre-XEN cell line X42. Shown are eight pairwise combinations of intrinsic (indicated with an asterisk after GFP) green fluorescence from the gene-targeted *PDGFRa* locus and immunofluorescence (magenta), together with DAPI (blue). Cells are immunoreactive for XEN markers GATA4, GATA6, SOX7, SOX17, and DAB2. But cells are negative for ES cell markers OCT4 and NANOG, and for TS cell marker CDX2.

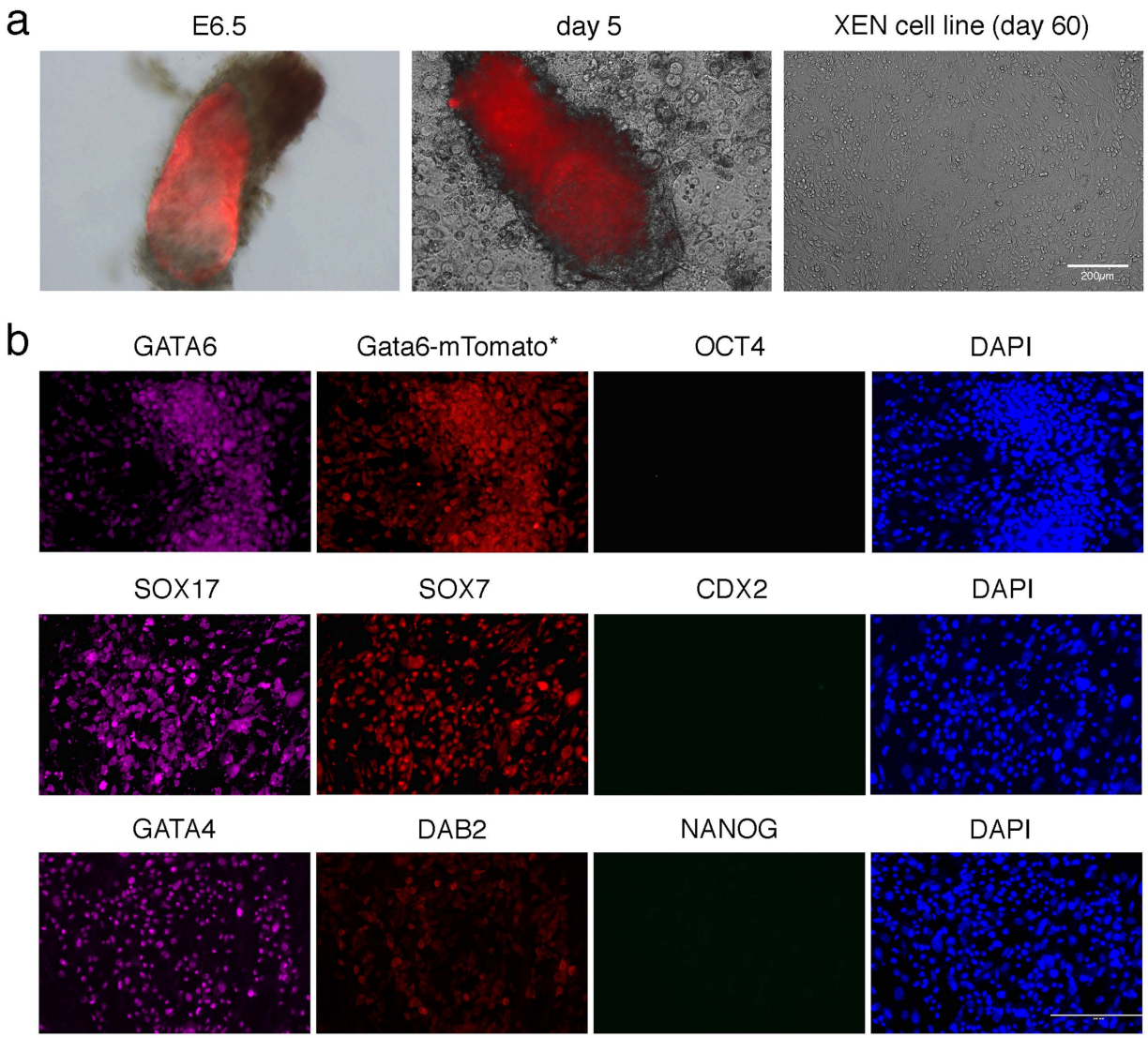

Figure 2. Derivation of post-XEN cell lines from whole E6.5 embryos. (**a**) Gata6-mTomato × Cdx2-GFP embryo and post-XEN cell line X-E6.5-82278-4. From left to right: whole E6.5 embryo, large outgrowth of embryo after 5 days in culture, and established post-XEN cell line on day 60. The embryo and the outgrowth display intrinsic red fluorescence of mTomato, but the expression of mTomato in the post-XEN cell line is below the detection level of intrinsic red fluorescence from mTomato. (**b**) Immunofluorescence analysis of post-XEN cell line X-E6.5-82278-4. Expression of mTomato from the *Gata6* promoter in the transgene is detectable with an antibody for RFP (first row, second column). Cells are immunoreactive (magenta) for XEN markers GATA4, GATA6, SOX7, SOX17, and DAB2. But cells are negative for ES cell markers OCT4 and NANOG, and for TS cell marker CDX2. Right-most column shows DAPI (blue).

Sox17-Cre male[34], and one hemizygous Gata6-mTomato female[36] mated with a homozygous Cdx2-GFP male[37] (Table 1). Xist1loxGFP is a GFP-containing targeted mutation in the *Xist* locus on the X-chromosome; Sox17 and Gata6 are XEN-cell markers; and Cdx2 is a marker for trophoblast stem cells. We removed the ectoplacental cone of the embryos as much as possible, and transferred each embryo separately into a well of 4-well dish coated with 0.1% gelatin and covered with MEF in TS cell medium including 25 ng/ml FGF4 and 1 μg/ml heparin (referred to as F4H). One day later, the embryos had attached to the surface and started to form an outgrowth. The embryos had formed a large outgrowth after 5 days. We used TrypLE Express to disaggregate the outgrowths and passaged cells into a well of a 4-well dish. After cells reached 70–80% confluency, they were passaged into a well of a 12-well dish. After they reached 70–80% confluency again, cells were passaged into a well of a 6-well dish, and we then obtained stable post-XEN cell lines. The intrinsic red fluorescence of mTomato produced from the *Gata6* promoter in the transgene was sufficiently high to detect it in the whole embryo and outgrowth, but not in the established post-XEN cell line at day 60 (Fig. 2a). We thus derived, using the whole-embryo method, a total of 30 post-XEN cell lines from 38 E6.5 embryos, at a 79% success rate (Table 1).

We found that mTomato expressed from the *Gata6* transgenic promoter in these cells can be detected by immunofluorescence with antibodies against RFP, together with GATA6 expressed from the endogenous *Gata6*

locus with antibodies against GATA6 (Fig. 2b). As was the case for our pre-XEN cell lines, our post-XEN cell lines are positive for XEN cell markers GATA4, GATA6, SOX7, SOX17, and DAB2 but negative for ES cell markers OCT4 and NANOG, and negative for TS cell marker CDX2.

Derivation of post-XEN cell lines from disaggregated E6.5 embryos. During the process of post-XEN cell line derivation from whole E6.5 embryos, we observed that some embryos had difficulty forming large outgrowths. We reasoned that some unidentified cell types in these embryos may inhibit XEN cell growth. We therefore proceeded to derive post-XEN cell lines from disaggregates of these embryos. We isolated 30 E6.5 embryos from three types of natural matings: a homozygous R26-tauGFP41 female with a heterozygous Sox17-Cre male[36], a heterozygous ROSA26-STOP-taulacZ female with a heterozygous Sox17-Cre male, and a wild-type CD1 female with a heterozygous PDGFRa-GFP male[28] (Table 1). We prepared disaggregates (Fig. 3a) by exposing the embryos to collagenase and deoxyribonuclease followed by TrypLE Express, and then gently pipetting the embryos in a glass pipette with a diameter of 50–60 μm. We plated the cell suspension, which consisted of a mixture of single cells and clumps of cells, from one disaggregated embryo separately into a well of 4-well dish coated with 0.1% gelatin and covered with MEF in standard TS medium including F4H. On day 3, XEN-like cells colonies appeared. We picked these colonies, disaggregated them by a glass pipette or by TrypLE Express for 5 min at 37 °C, and passaged them into a well of a 4-well dish. (We did not succeed in deriving post-XEN cell lines from single GFP+ cells that we placed in wells of a 96-well dish.) We thus derived, using the disaggregation method, a total of 30 post-XEN cell lines from 30 E6.5 embryos, at a 100% success rate (Table 1).

As was the case with our pre-XEN cell lines and post-XEN cell lines derived from E6.5 whole embryos, cells are immunoreactive for XEN markers GATA4, GATA6, SOX7, SOX17, and DAB2, but negative for ES cell markers OCT4 and NANOG, and negative for TS cell marker CDX2 (Fig. 3b). Thus, we increased the success rate of post-XEN cell derivation from 79% for whole E6.5 embryos to 100% for disaggregated E6.5 embryos.

Derivation of post-XEN cell lines from E5.5 whole embryos. In our final set of experiments, we derived post-XEN cell lines from 5.5 day-old embryos. We isolated 17 embryos from 33 E5.5 implantation sites from a mating of three homozygous R26-tauGFP41 females with three heterozygous Sox17-Cre males, and a mating of a homozygous ROSA-STOP-taulacZ female with a heterozygous Sox17-Cre male (Table 1). We removed as much of the ectoplacental cone as possible, and transferred one whole embryo separately into a well of a 4-well dish coated with 0.1% gelatin and covered with MEF, in TS medium including F4H (Fig. 4a). In our experience, the less ectoplacental cone and extraembryonic ectoderm remains in a dissected embryo, the easier it is to derive a post-XEN cell line. We surmise that ectoplacental cone cells and extraembryonic ectoderm cells (trophoblast-derived cells) compete with or inhibit XEN cells in culture. For 14/17 embryos, large outgrowths had appeared by day 5. We disaggregated these outgrowths with TrypLE Express on day 7, and passaged the cells into a well of a 4-well dish. On day 11, XEN-like colonies had accumulated. We picked these colonies, combined them, disaggregated them with a glass pipette or with TrpLE Express for 5 min at 37 °C, and passaged them into a well of a 4-well dish. We thus established 14 post-XEN cell lines after ~21 days in culture. For the remaining 3/17 embryos, trophoblast-derived cells surrounded the outgrowth. We performed a disaggregation by pipette on day 3, and switched to ES medium with LIF. When XEN cells became abundant, we passaged the cells onto gelatin-coated dishes without MEF, and switched back to TS medium including F4H. Three post-XEN cell lines were established after ~50 days culture. We thus derived a total of 17 post-XEN cell lines from 17 E5.5 embryos, at a 100% success rate (Table 1).

These post-XEN cell lines are immunoreactive for XEN cell markers GATA4, GATA6, SOX7, SOX17, and DAB2, but negative for ES cell markers OCT4 and NANOG, and negative for TS cell marker CDX2 (Fig. 4b).

Post-XEN cell morphology, population characteristics, and *in vitro* differentiation. Like pre-XEN cell lines[10], our cultures of post-XEN cell lines contain at least two cell morphologies: a rounded, highly refractile cell type and a more stellate epithelial-like cell type (Fig. 5a). At higher densities, post-XEN cells can form epithelial sheets (Fig. 5b) and often a lattice-type structure (Fig. 5c). To determine if these two cell morphologies represent two cell types in the cultures, we FACS-sorted single GFP+ post-XEN cells (X-E6.5-Z0617-5) directly into wells of a 96-well dish, and derived two subclones. Cells of these subclones continued to exhibit either round or epithelial-like cell morphologies (Fig. 5d).

To evaluate the population characteristics of post-XEN cell lines, we analyzed three GFP-expressing cell lines (X-E6.5-Z0617-5, X-E6.5-Z0617-2 and X-E5.5-10) by immunofluorescence for GATA4 and counterstaining with DAPI. Sets of fluorescence images were captured for each line visualizing the intrinsic fluorescence of GFP, DAPI, and GATA4 immunoreactivity (Fig. 5e). The images were evaluated with a custom CellProfiler pipeline that segmented cells using the DAPI fluorescent signal. A cutoff for size and nuclear roundness was used to exclude the MEF population as much as possible. We then evaluated each cell for levels of GFP and GATA4 signal. In these three post-XEN cell lines, >94% of cells evaluated exhibited GFP fluorescence and are GATA4+ (Fig. 5f). The populations of cells that expressed either GFP or GATA4 but not both represented 0–3% of the cells evaluated. The remaining population of cells, which expressed neither GFP nor GATA4, may reflect another cell type, or MEFs that escaped exclusion during the cell identification step in the custom CellProfiler pipeline.

We asked if post-XEN cells can differentiate into a VE identity by incubation with BMP4[38,39]. We cultured four post-XEN cell lines (X-E5.5-9, X-E6.5-Z0617-2, X-E6.5-Z0617-5 and X-E6.5-78097-7) and three pre-XEN cell lines (X42, X47, X-ICM-4) in gelatin-coated dishes in TS medium with F4H, plus 10 ng/ml BMP4. In parallel, we cultured the same cell lines in TS medium with F4H without BMP4. Four days later, we performed immunofluorescence for E-cadherin, a VE marker. We found that culture with BMP4 induces expression of E-cadherin in post-XEN cells, as well as in pre-XEN cells[10] (Fig. 5g-h).

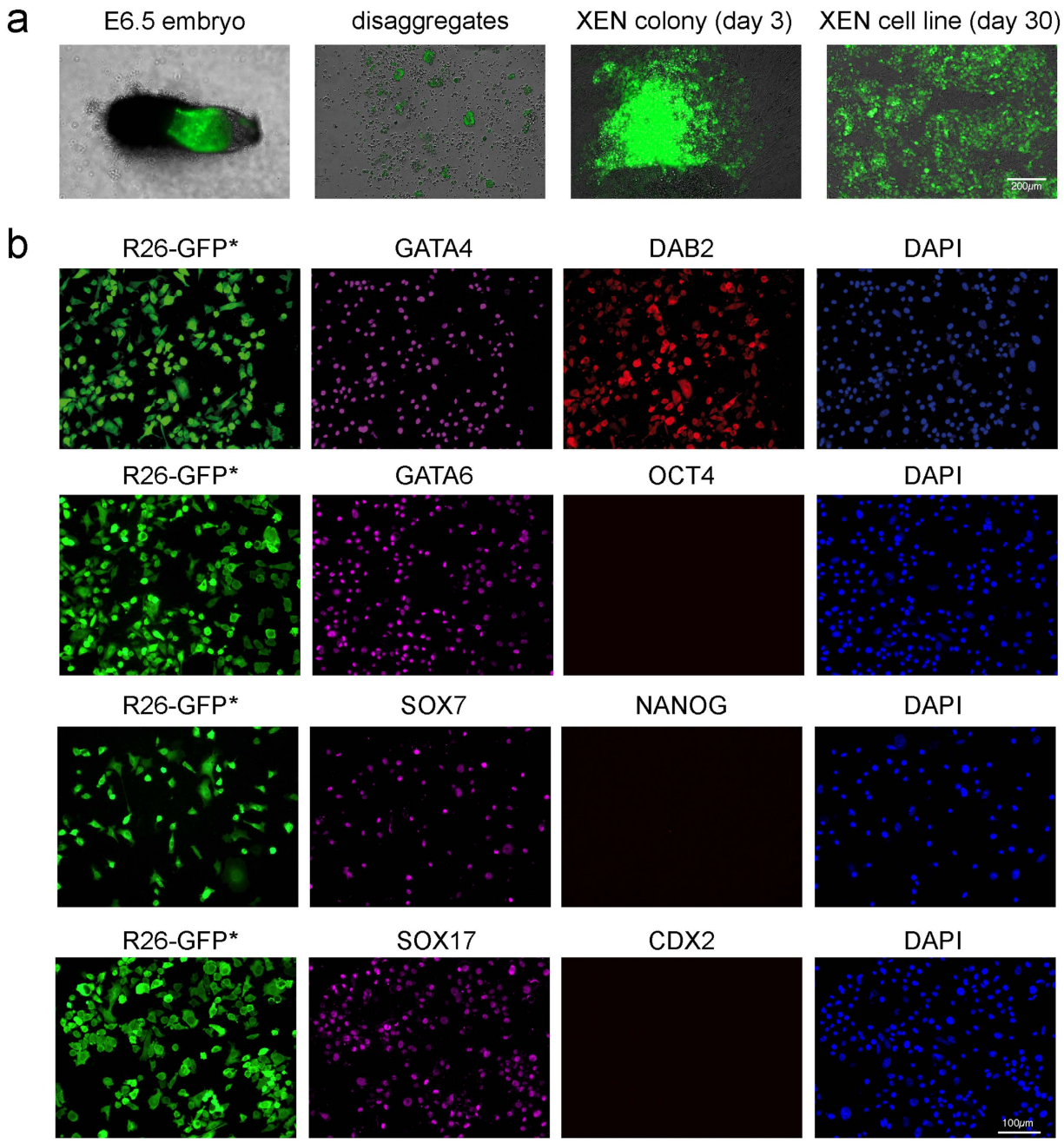

Figure 3. Derivation of post-XEN cell lines from disaggregates of E6.5 embryos. (**a**) R26-tauGFP41 × Sox17-Cre E6.5 embryo and post-XEN cell line X-E6.5-Z0617-2. From left to right: whole E6.5 embryo, disaggregated embryo, XEN-like colony expressing GFP on day 3 of culture, and established post-XEN cell line on day 30. Intrinsic green fluorescence of GFP. (**b**) Fluorescence analysis of post-XEN cell line X-E6.5-Z0617-5. First column: intrinsic (indicated with an asterisk after GFP) green fluorescence of GFP expressed from the *ROSA26* locus after activation by Cre recombinase that is expressed from the gene-targeted *Sox17* locus. Second column: cells are immunoreactive (magenta) for XEN markers GATA4, GATA6, SOX7, and SOX17. Third column: cells are immunoreactive for XEN marker DAB2, but negative for ES cell markers OCT4 and NANOG, and negative for TS cell marker CDX2. Fourth column: DAPI (blue).

NanoString gene expression analyses of XEN and ES cell lines. We applied the NanoString multiplex platform for gene expression[40–42] and agglomerative clustering, in order to compare the patterns of expression of selected genes in four pre-XEN cell lines, three post-XEN cell lines, and three ES cell lines that we had derived in other experiments (Fig. 6). We find that all our XEN cell lines have high levels of expression of XEN-specific genes, such as *Dab2, Gata4, Gata6, Pdgfra, Sox7,* and *Sox17,* versus low or no expression of ES cell-specific genes such as *Nanog, Pou5f1/Oct4, Sox2,* and *Nr0b1*. There is no expression of EpiSC-specific genes

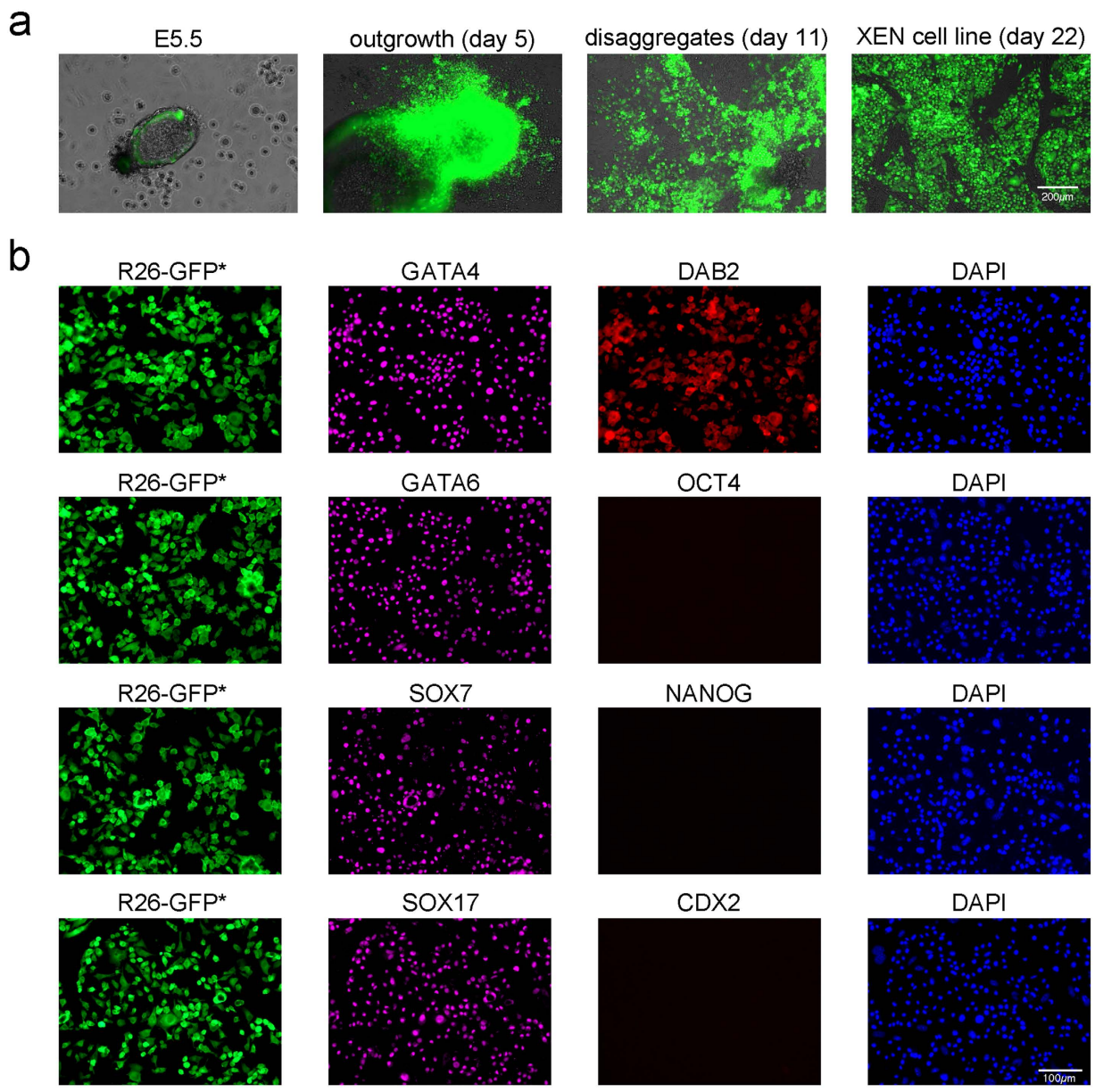

Figure 4. Derivation of post-XEN cell lines from whole E5.5 embryos. (**a**) Sox17-Cre × R26-tauGFP41 E5.5 embryo and post-XEN cell line. From left to right: whole E5.5 embryo, large outgrowth of embryo after 5 days in culture, cells after outgrowth disaggregation and culture for 11 days, and established post-XEN cell line X-E5.5-8 on day 22. Combined intrinsic green fluorescence of GFP/bright-field image. (**b**) Fluorescence analysis of post-XEN cell line X-E5.5-10. First column: intrinsic (indicated with an asterisk after GFP) green fluorescence of GFP from the *ROSA26* locus after activation by Cre recombinase that is expressed from the gene-targeted *Sox17* locus. Second column: cells are immunoreactive (magenta) for XEN markers GATA4, GATA6, SOX7, and SOX17. Third column: cells are stained for XEN marker DAB2, but not for ES cell markers OCT4 and NANOG, and not for TS cell marker CDX2. Fourth column: DAPI (blue).

such as *Cer1* and *Fgf5* (data not shown). Thus, the NanoString gene expression analysis confirms and extends the immunofluorescence profiles.

Post-XEN cells contribute to the extraembryonic endoderm of chimeras after injection into blastocyts. A final and stringent test of the potency of stem cells is to assess their contribution to embryonic and extraembryonic tissues *in vivo*. We injected into blastocysts (B6D2F2, C57BL/6 J, or CD1) cells of two R26-tauGFP41+ pre-XEN cell lines (X-ICM-4 and X-ICM-5) and one PDGFRa-GFP+ pre-XEN cell line (X47) that we had derived from isolated ICMs or blastocysts, and cells from four R26-tauGFP41+ post-XEN cell lines (X-E6.5-Z0617-2, X-E6.5-Z0617-5, X-E5.5-6 and X-E5.5-9) and one PDGFRa-GFP+ post-XEN cell line (X-E6.5-Z0663-9) that we had derived from E5.5 embryos or disaggregated E6.5 embryos (Table 2). For pre-XEN cells, we identified 3 chimeras among 8 E6.5 embryos (38%), and 15 chimeras among 43 E7.5 embryos (35%),

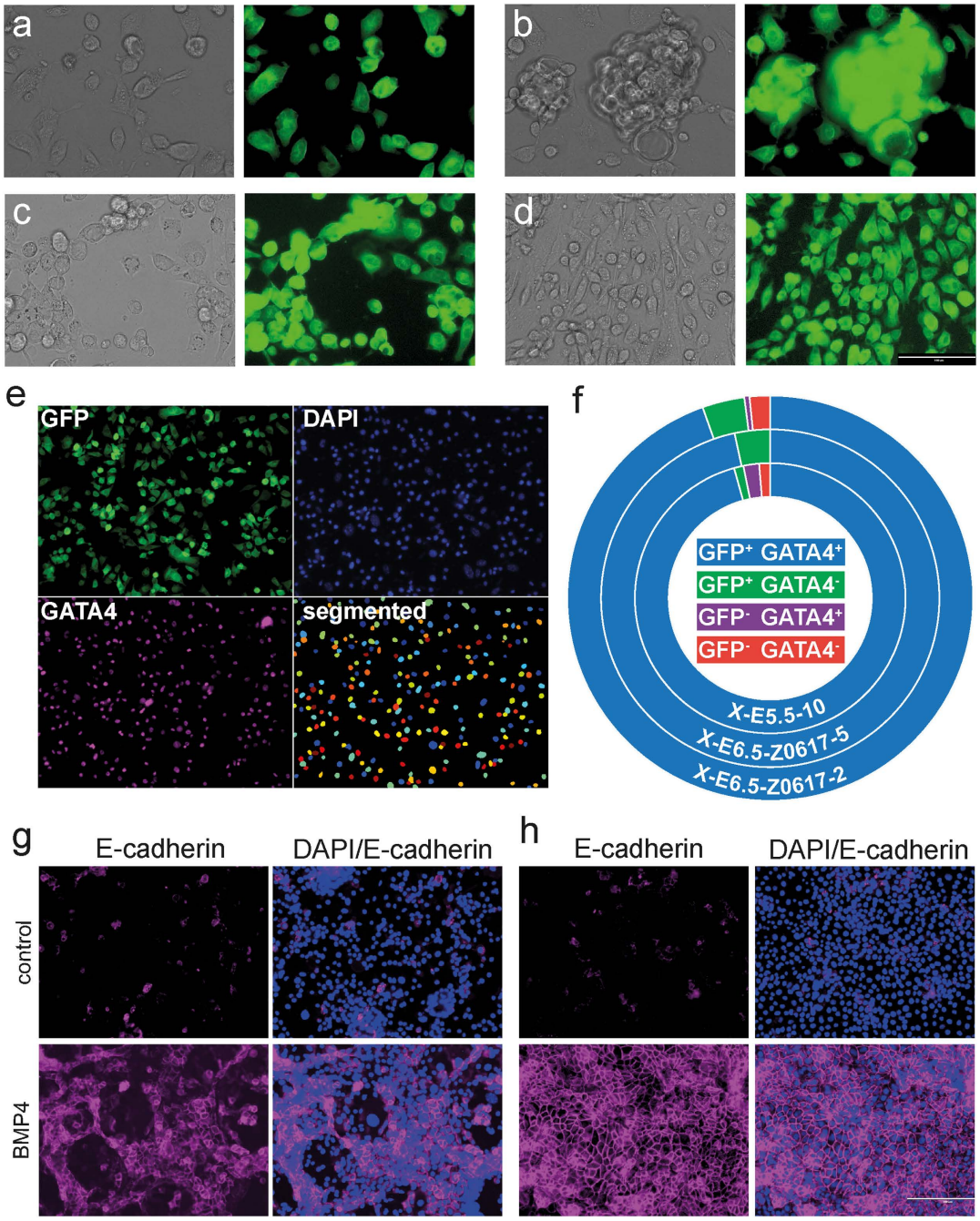

Figure 5. Post-XEN cell morphology in culture, population characteristics, and *in vitro* differentiation with BMP4. (**a**) Post-XEN cells X-E6.5-Z0617-5 cultured at low density contain a refractile, round cell type, and an epithelioid cell type. (**b**) Post-XEN cells X-E6.5-Z0617-5 cultured to near-confluency show the presence of individual cells and an epithelial sheet of cells. (**c**) Post-XEN cells X-E6.5-Z0617-5 participate in a lattice-type structure. (**d**) A subcloned cell line of X-E6.5-Z0617-5 continues to contain a refractile, round cell type, and an epithelioid cell type. (**e**) Analysis of population characteristics of the post-XEN cell lines X-E5.5-10, X-E6.5-Z0617-5, and X-E6.5-Z0617-2. Representative images of fields of view used to analyze Z0617-5 are shown. The GFP signal reflects intrinsic fluorescence, the GATA4 signal reflects immunoreactivity, and DAPI was used to counterstain the nuclei. The bottom right image shows the segmented nuclei of cells selected for marker analysis after the DAPI signal had undergone thresholding, declumping, and filtering for size and nuclear roundness. The individual cells are shown in multiple colors to clearly show the distinct cells that were used for analysis. Three sets of images were evaluated per cell line, and 500–1000 cells were evaluated per cell line. (**f**) Marker characteristics of the three cell lines are shown in the form of a doughnut chart, as determined by intrinsic fluorescence of GFP and immunoreactivity for GATA4. Cells are divided into four populations. (**g**,**h**) Immunofluorescence analysis of pre-XEN cell line X47 (**g**) and post-XEN cell line X-E6.5-Z0617-5 (**h**). Expression of the VE marker E-cadherin is higher after treatment with 10 ng/ml BMP4, in gelatin-coated plates. Left columns: E-cadherin (magenta). Right columns: DAPI signal (blue) merged with E-cadherin (magenta).

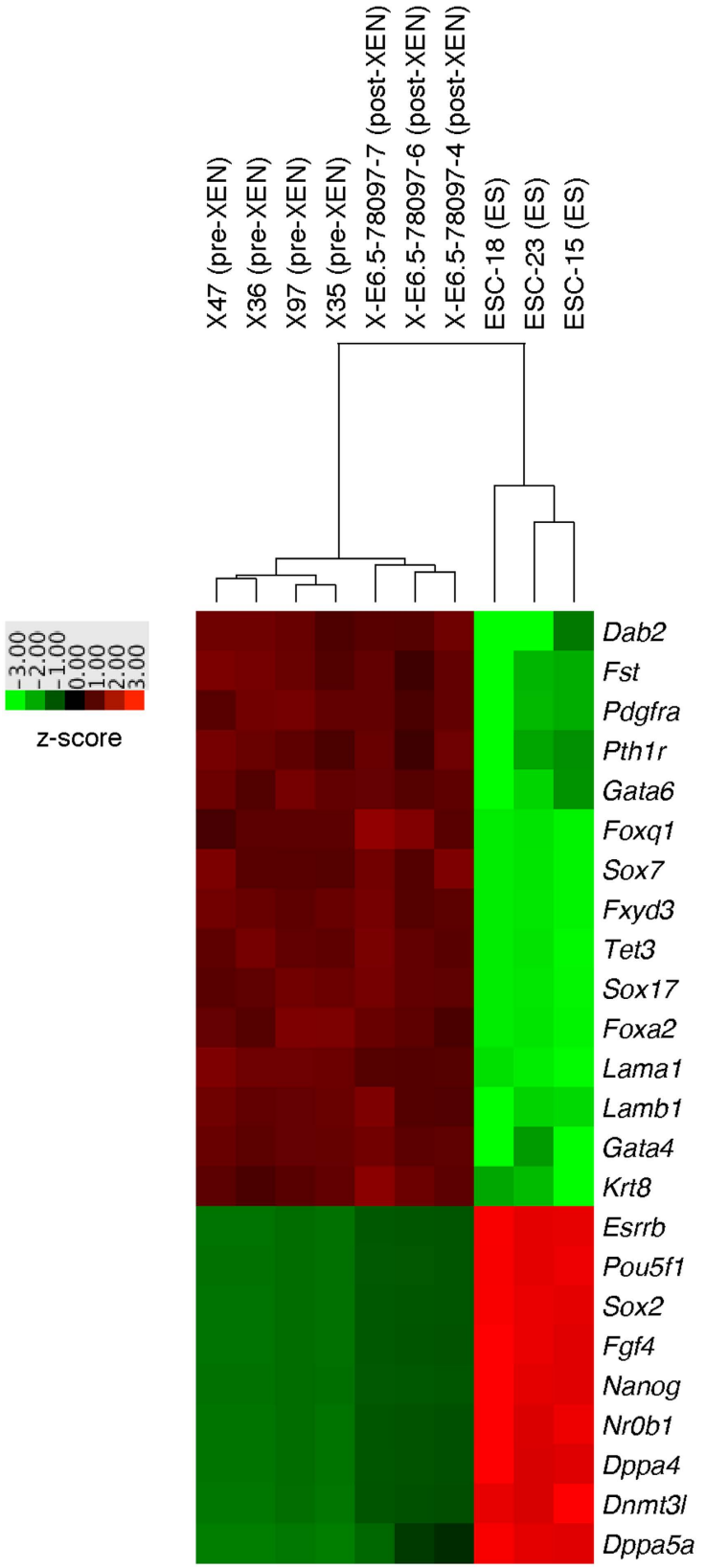

Figure 6. NanoString gene expression analyses of XEN and ES cell lines. Heatmap analysis of four pre-XEN cell lines, three post-XEN cell lines, and three ES cell lines. Red means high expression level, green means lowly expression level. Agglomerative clustering was performed with nSolver software. Among the classical XEN cell markers are *Dab2, Pdgfra2, Gata6, Sox7, Sox17,* and *Gata4.* Among the classical ES cell markers are *Oct4/Pou5f1, Sox2, Ffg4,* and *Nanog*. Pre-XEN and post-XEN cell lines share a gene expression profile that is typical for XEN cell lines.

Cell lines	Strains	No. Blastocysts injected	No. Blastocysts transferred	No. Implantation sites	No. E6.5 embryos	No. E6.5 chimeras	No. E7.5 embryos	No. E7.5 chimeras
Pre-XEN cells								
X-ICM-4	R26-tauGFP41 × Sox17-Cre	59	59	53	8	3	33	10
X-ICM-5	R26-tauGFP41 × Sox17-Cre	15	15	12			8	3
X47	PDGFRa-GFP× CAG::mRFP1	8	8	3			2	2
Sum		82	82	68	8	3	43	15
Post-XEN cells								
X-E6.5-Z0617-2	R26-tauGFP41 × Sox17-Cre	31	31	24	13	6	10	2
X-E6.5-Z0617-5	R26-tauGFP41 × Sox17-Cre	83	83	68	11	4	46	19
X-E6.5-Z0663-9	PDGFRa-GFP × CD1	15	15	10	4	1	5	1
X-E5.5-6	Sox17-Cre × R26-tauGFP41	22	22	16	13	6		
X-E5.5-9	Sox17-Cre × R26-tauGFP41	45	45	38	8	2	22	8
Sum		196	196	156	49	19	83	30

Table 2. Post-XEN cell lines contribute to extraembryonic endoderm in chimeras.

from a total of 82 injected blastocysts (18/82 = 22%). For post-XEN cells, we identified 19 chimeras among 49 E6.5 embryos (39%) and 30 chimeras among 83 E7.5 embryos (36%), from a total of 196 injected blastocysts (49/196 = 25%). The percentages of identifiable chimeras among embryos and with regard to injected blastocysts are thus comparable for pre-XEN and post-XEN cell lines.

We found that pre-XEN and post-XEN cells contribute to extraembryonic endoderm at E6.5 or E7.5 (Fig. 7), as is typical for XEN cells injected into blastocysts[10,20]. We sectioned the decidua of E6.5 or E7.5 embryos obtained by injecting pre-XEN or post-XEN cells into blastocysts, and performed immunofluorescence with antibodies against PDGFRa and E-cadherin. PDGFRa is expressed in the PE and VE[32], and E-cadherin is expressed in the VE and epiblast[16,43,44]. Therefore, extraembryonic endoderm cells that are only immunoreactive for PDGFRa are PE, and cells that are immunoreactive for both PDGFRa and E-cadherin are VE. We analyzed 103 GFP+ cells in E6.5 chimeras obtained by injection of X-ICM-4 (pre-XEN), and 123 GFP+ cells in E7.5 chimeras with X-ICM-5 (pre-XEN), and found that all 226 GFP+ cells reside within the PE region and are immunoreactive for PDGFRa but not for E-cadherin; thus, no GFP+ cell contributed to the VE (Fig. 7a–c). We analyzed 75 GFP+ cells in E6.5 chimeras and 142 GFP+ cells in E7.5 chimeras obtained by injection of X-E6.5-Z0617-5 (post-XEN), and found that all 217 GFP+ cells reside within the PE region and are immunoreactive for PDGFRa but not for E-cadherin; thus, no GFP+ cell contributed to the VE (Fig. 7d–k).

Discussion

We report here that XEN cell lines can be derived efficiently from postimplantation embryos, and from a wide variety of strains and crosses. We believe that it is prudent to continue to refer to these two types of cell lines with regard to the embryonic stage from which they were derived. Additional work will be necessary to identify and characterize possible differences, subtle or substantial, between pre-XEN and post-XEN cell lines.

With our E5.5 whole-embryo method and E6.5 disaggregated-embryo method, we attained a success rate of 100%: we derived 17 post-XEN cell lines from 17 embryos and 30 post-XEN cell lines from 30 embryos, respectively. There are only ~11 PrE cells at the late blastocyst stage[45]. As E5.5 and E6.5 embryos contain ~95 and ~250 extraembryonic endoderm cells respectively[46], these higher numbers of potential source cells may explain the 100% efficiency of XEN cell line derivation from postimplantation embryos. Other, experimental, reasons for the highly efficient derivation may be the reduction of the negative effects of trophoblast-derived cells on XEN cells in the disaggregated E.6 embryo method, and the promotion of XEN cells over trophoblast-derived cells by culturing in ES medium with LIF in the whole-embryo E5.5 embryo method. Regardless of these numerical and experimental explanations, a more exciting, and biological, explanation is that extraembryonic tissues preserve developmental plasticity through implantation.

Since the first report of XEN cell derivation from blastocysts in 2005 (ref. 10), there are three types of methods in place to derive XEN cell lines, either directly from blastocysts[10,20] or by conversion from ES cells[20–26] or, recently, by reprogramming from fibroblasts[27]. The success rate for derivation of XEN cell lines from blastocysts was 21% in TS medium including F4H and 56% in ES medium supplemented with LIF[20], which is identical to our success rate of 57% in ES medium supplemented with LIF. Recently, LIF has been reported to support PrE expansion during pre-implantation embryo development[47], and LIF could also be supporting XEN cell expansion. The conventional protocol to derive ES cell lines from blastocysts involves also ES medium supplemented with LIF[48,49]; this medium can be used to derive XEN cell lines from blastocysts, by picking XEN cell colonies or by removing ES cell colonies, but there is always the risk of deriving ES cell lines[50]. The indirect generation of XEN cell lines from ES cell lines requires, obviously, the prior generation or availability of such lines.

TS cell lines can be derived from the extraembryonic ectoderm of postimplantation embryos in TS medium including FGF4 and heparin[9,11]. Interestingly, we did not derive TS cell lines from either whole or disaggregated embryos using this medium. We did observe transiently cells and colonies with a morphology that is consistent with TS cells or EpiSCs, but they disappeared from the culture with time. In our immunofluorescence analyses, our XEN cell lines do not contain cells that express the trophectoderm marker Cdx2, and our Nanostring analyses do not reveal expression of TS-specific genes either. The cell lines that we derived from postimplantation

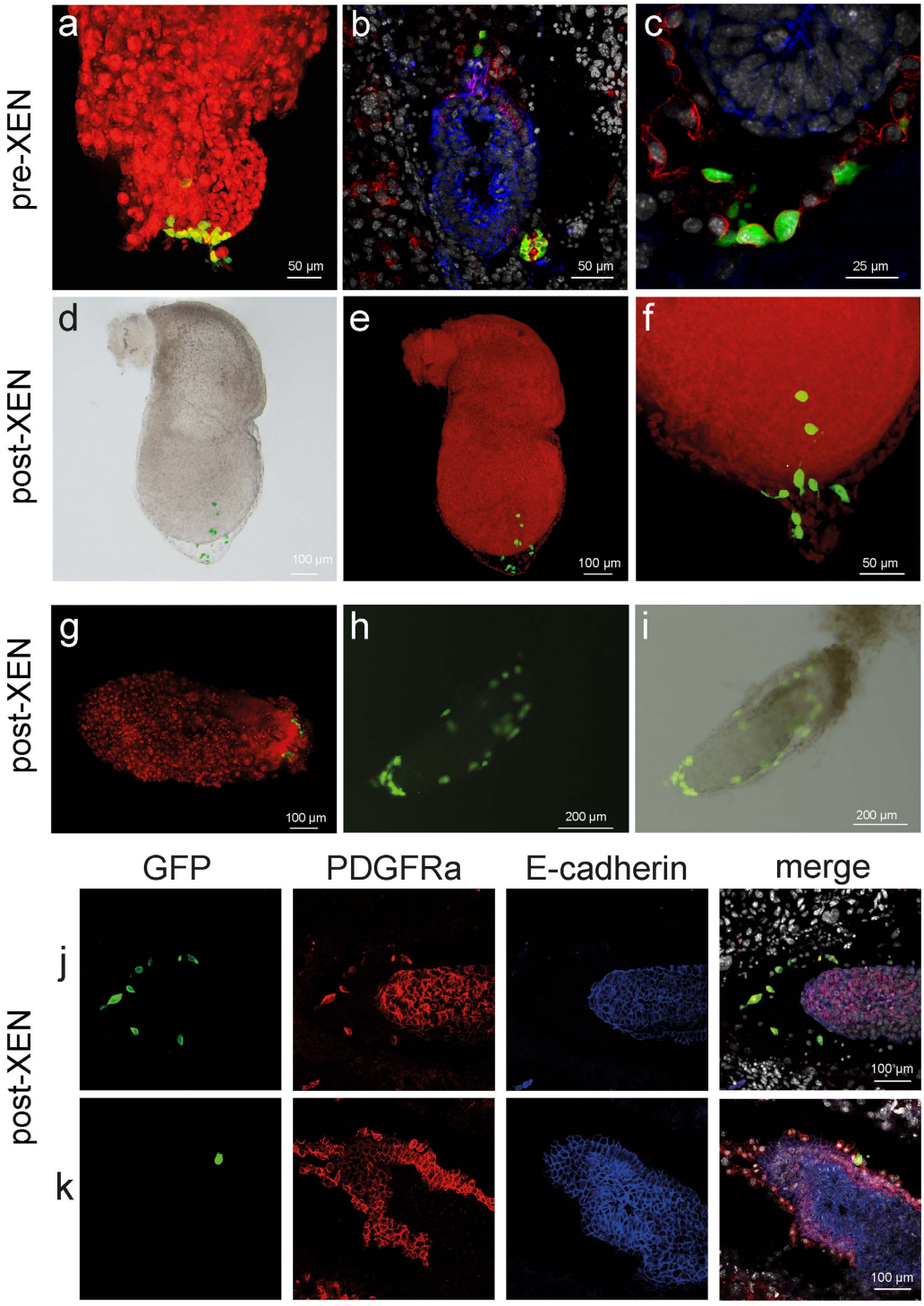

Figure 7. XEN cells contribute to the parietal endoderm of chimeric embryos. (**a–c**) Pre-XEN cell line X-ICM-4 contributes to the PE of E6.5 and E7.5 embryos. GFP+ cells are derived from the injected pre-XEN cells. (**a**) Wholemount of E7.5 embryo. (**b,c**) Sections of the decidua of E6.5 embryos, showing the merged image of immunofluorescence for PDGFRa (red) and E-cadherin (blue), and fluorescence of DAPI (white) and GFP (green). (**d–k**) Post-XEN cell lines (X-E5.5-9, X-E6.5-Z0617-5, and X-E6.5-Z0617-2) contribute to the PE of E6.5 or E7.5 embryos. (**d–f**) Wholemount. GFP+ cells derived from X-E5.5-9 contribute to an E7.5 embryo. (**g**) Wholemount. GFP+ cells derived from X-E6.5-Z0617-5 contribute to an E7.5 embryo. (**h,i**) Wholemount. GFP+ cells derived from X-E6.5-Z0617-2 contribute to an E6.5 embryo. (**j,k**) Sections of the decidua of E7.5 embryos containing GFP+ cells derived from X-E6.5-Z0617-5, with immunofluorescence for PDGFRa (red) and E-cadherin (blue), fluorescence of DAPI (white) and GFP (green), and merge. The single GFP+ cell in (**k**) apposes but does not belong to the VE, as it is not immunoreactive for E-cadherin. (**a–g**) and (**j,k**) were imaged using a Zeiss LSM 710 confocal microscope, and (**h–i**) were imaged using a Nikon SMZ25 stereofluorescence microscope. In (**a**) and (**e–g**) the red counterstain is propidium iodide.

embryos in TS medium are thus not TS cell lines, not ES cell lines, and not EpiSC lines, but represent XEN cell lines. Why then are XEN cell lines obtained rather than TS cell lines with our method, which cultures the cells in the same TS medium? The protocol for deriving TS cell lines from postimplantation embryos prescribes that the extraembryonic ectoderm be dissected out and that the embryo and the VE and PE be discarded[12]. By contrast, we remove the ectoplacental cone from the embryo. We speculate that the VE and PE surrounding the extraembryonic ectoderm inhibit TS cell growth, and/or that VE- and PE-derived cells divide at a faster rate than TS cells and dominate with time the cell culture.

Chemical reprogramming of mouse fibroblasts to the iPS cells involves a XEN-like intermediary stage as a bridge between somatic and pluripotent cells[19]. In another study, iXEN clones arise in parallel to iPS clones during OSKM-mediated reprogramming of mouse fibroblasts[27]. More knowledge about the derivation, biology, culture, and conversion of XEN cell lines may be beneficial to develop robust and efficient protocols for the derivation of iPS cell lines by chemical reprogramming with small molecules.

In future experiments, it will be interesting to determine the cell type(s) that are at the origin of the post-XEN cell lines.

Methods

Mouse strains. The PDGFRa-GFP strain[28] was obtained from The Jackson Laboratory (JAX), strain 7669, strain name B6.129S4-Pdgfra<tm11(EGFP)>Sor/J. The CAG::mRFP1 strain[29] was obtained from JAX, strain 5645, strain name Tg(CAG-mRFP1)1F1Hadj/J. The D4/XEGFP strain[30] was obtained from JAX, strain 3116, strain name Tg(CAG-EGFP)D4Nagy/J. The Sox17-Cre strain[34] was obtained from MMRRC, strain 036463-UNC, strain name Sox17<tm2(EGFP/cre)Mgn>/Mmnc. (Although this strain is reported to contain and express GFP, and we confirm the presence of GFP in this targeted insertion in the *Sox17* locus, we cannot detect GFP expression in embryos and cell lines derived from them.) The Xist1loxGFP strain[35] was provided by the RIKEN BioResource Center through the National BioResource Project of the MEXT (Japan), strain RBRC01260, strain name B6;129(Cg)-Xist<tm2Sado>. The GATA6-mTomato strain[36] was provided by the RIKEN BioResource Center, strain RBRC04900, strain name B6;B6C3(129)-Tg(GATA6-mTomato)3Hmd. The Cdx2-GFP strain[37] was obtained from JAX, strain 18983, strain name Cdx2<tm1Yxz>/J. The ROSA26-STOP-taulacZ reporter strain[51] was generated by Dr. Ivan Rodriguez in the laboratory of P.M. at The Rockefeller University, and is publicly available from JAX, strain 6744, STOCK Gt(ROSA)26Sor<tm1.1Mom>/MomJ. The R26-tauGFP41 reporter strain[52] was a gift from Dr. Uli Boehm, Universität des Saarlandes, Homburg, Germany. For the preparation of mouse embryonic fibroblasts, strain 3208, strain name Tg(DR4)1Jae was obtained from JAX.

TS cell medium. Advanced RPMI-1640 (Gibco, #12633-012) was supplemented with 20% (vol/vol) FBS (HyClone, #SH30071.03), 2 mM GlutaMAX Supplement (Gibco #35050), 1% penicillin/streptomycin (Specialty Media #TMS-AB2-C), 0.1 mM β-mercaptoethanol (Gibco #21985-023), 1 mM sodium pyruvate (Gibco #11360-039), supplemented with 25 ng/ml FGF4 (Peprotech #100-31) and 1 μg/ml heparin (Sigma #H3149).

ES cell medium. ES cell lines were maintained on MEF-coated, pregelatinized tissue culture dishes (Falcon) in DMEM (Specialty Media #SLM-220) supplemented with 15% FBS (HyClone #SH30071.03), 2 mM GlutaMAX Supplement, 1% penicillin/streptomycin, 1% β-mercaptoethanol (Specialty Media #ES-007-E), 0.1 mM nonessential amino acids (Gibco #11140-035), 1 mM sodium pyruvate, and 1000 IU/ml leukemia inhibitory factor (LIF) (Millipore #ESG1107).

Derivation of ES cell lines. Embryos were collected at the 2–8 cell stage by flushing oviducts using M2 medium (Sigma #M7167), and cultured in KSOM medium (Millipore #MR-106-D) to the blastocyst stage. The zona pellucida of blastocysts was then removed using acid Tyrode solution (Sigma #T1788). Blastocysts were transferred separately into a well of a 96-well dish (Falcon) coated with 0.1% gelatin (Specialty Media #ES-006-B) and covered with MEF in ES medium supplemented with LIF and 1 μM PD0325901 (Axon #1408) and 3 μM CHIR99021 (Axon #1386), a combination of chemicals that is typically referred to as "2i". After 4 days TrypLE Express (Gibco #12604-013) was used to disaggregate the embryonic outgrowths, and cells were passaged to a well of 24-well dish to derive ES cell lines.

Derivation of pre-XEN cell lines from blastocysts. Embryos were collected at the 2–8 cell stage embryos and cultured in in KSOM medium to the blastocyst stage. The zona pellucida of blastocysts was removed using acid Tyrode solution. Blastocysts were transferred separately into a well of a 4 well-dish (Nunc #176740) coated with 0.1% gelatin and covered with MEF in ES medium supplemented with LIF. The XEN lines were derived as described[20].

Derivation of post-XEN cell lines from whole E5.5 or E6.5 embryos. The ectoplacental cone was removed with forceps or needles. A whole embryo was placed in a well of a 4-well dish (Nunc #176740) coated with 0.1% gelatin and covered with MEF in TS medium including F4H. After the embryos formed a large outgrowth, TrypLE Express was used to disaggregate the outgrowths and passage cells into a 4-well dish. When cells reached 70–80% confluency, they were passaged into a well of a 12-well dish until XEN cell lines were obtained, which were then passaged into a well of a 6-well dish. If the outgrowth of an E5.5 embryo grew well and XEN cells thrived, we continued to culture cells in TS medium including F4H. But if the outgrowth grew slowly and XEN cells were surrounded by trophoblast-derived cells, we cultured cells in ES medium supplemented with LIF, culture conditions that would inhibit trophoblast-derived cells; when XEN cells started to become abundant, we switched to TS medium including F4H. To prepare XEN cells for RNA extraction, cells were cultured in dishes coated with 0.1% gelatin but without MEF in TS medium including F4H.

Derivation of post-XEN cell lines at E6.5 from disaggregated embryos. The ectoplacental cone was removed with forceps or needles. A whole embryo was treated with 0.1 mg/ml collagenase (Gibco #17104-019) and 0.01 mg/ml deoxyribonuclease (Gibco #D5025) for 20–30 min at room temperature, followed by 0.2 mg/ml TrypLE for 5 min at room temperature. The embryo was disaggregated into single cells using a glass pipette with a diameter of 50–60 μm. Cells were transferred into a well of a 4-well dish (Nunc #176740) coated with 0.1% gelatin and covered with MEF in TS medium including F4H. Three days later XEN colonies appeared. We picked these colonies, disaggregated them with a glass pipette or by TrypLE Express for 5 min at 37 °C, and passaged them into a well of a 4-well dish. When cells reached 70–80% confluency, they were passaged into a well of a 12-well dish until XEN cell lines were obtained, which were then passaged into a well of a 6-well dish.

Population characteristics. Images representing Fields of View of post-XEN cell lines were imaged for intrinsic fluorescence of GFP, fluorescence of DAPI, and GATA4 immunoreactivity. Automated cell population characteristics were determined with CellProfiler http://cellprofiler.org/ (Broad Institute, Cambridge, MA, USA). The DAPI signal was used to segment individual cells by thresholding, de-clumping, and applying size and roundness filter in an effort to evaluated single cells that are not MEFs. After filtering, the GFP and GATA4 signals were evaluated in each cell, and the population characteristics for these two markers were quantified.

***In vitro* differentiation.** Gelatin-coated plates were prepared by coating with 0.1% gelatin overnight at room temperature. XEN cells were cultured in TS medium with F4H and with or without 10 ng/mL BMP4 (Peprotech, 120–05) on gelatin-coated plates for four days.

Immunofluorescence and imaging. Cell lines X42, X44, X47 (PDGFRa-GFP × CAG::mRFP1); X35, X36 (B2D6F1 × D4/EGFP); X97, X107 (D4/EGFP × DBA2/N); X-E6.5-81346-8 (ROSA-STOP-taulacZ x Sox17-Cre); X-E6.5-Z0617-2, X-E6.5-Z0617-5, X-E6.5-Z0617-8 (R26-tauGFP41 x Sox17-Cre); X-E6.5-78097-4 (Xist1loxGFP × DBA/2 N); X-E6.5-82278-4 (Gata6-mTomato × Cdx2-GFP); X-E6.5-Z0663-12 (CD1 × PDGFRa-GFP); and X-E5.5-6, X-E5.5-8, X-E5.5-9, X-E5.5-10, X-E5.5-13 (R26-tauGFP41 × Sox17-Cre) were cultured in 4-well or 24-well dishes. Cells were fixed in 4% paraformaldehyde at 4 °C overnight or room temperature for 30 min, permeabilized with 0.1% Triton X-100 in 1× PBS (1× PBST) for 30 min and blocked with 5% normal donkey serum (Jackson ImmunoResearch Laboratories, #017-000-121) diluted in 1× PBST (blocking solution) for 1 hr. Primary antibodies were diluted at 1:50–1:200 in blocking solution and samples incubated at 4 °C rotating overnight. After three 10-min washes in 1× PBST 10 min, samples were incubated for 1–1.5 hr at room temperature in a 1:500 dilution of secondary antibody in blocking solution, then washed and covered with 1× PBST containing DAPI. Images were taken with an AMG EVOS (Life Technologies), Zeiss LSM 710 confocal microscope, and Nikon SMZ25 stereofluorescence microscope.

Primary antibodies from Santa Cruz Biotechnology were against GATA4 (#SC-1237), DAB2 (#SC-13982), OCT3-4 (#SC-5279), NANOG (#SC-376915), and CDX2 (#SC-166830). Primary antibodies from R&D Systems were against GATA6 (#AF1700), SOX7 (#AF2766), SOX17 (#AF1924), and PDGFRa (#AF1062). Primary antibodies against E-cadherin (ECCD2) were from Invitrogen (#13-1900). Secondary antibodies from Jackson ImmunoResearch Laboratories were Cy5™ AffiniPure Donkey anti-goat IgG (H+L) (#705-175-147), and Cy5™ AffiniPure Donkey anti-rabbit IgG (H+L) (#711-175-152). Secondary antibodies from Invitrogen were Donkey anti-rabbit IgG (H+L) with Alexa Fluor 546 (#A10040), Goat anti-rat IgG (H+L) with Alexa Fluor 546 (#A11081), Donkey anti-mouse IgG with Alexa Fluor 546 (#A10036), Donkey anti-mouse IgG with Alexa Fluor 488 (#A21202), and Goat anti-chicken IgY (H+L) with Alexa Fluor 488 (#A11039).

NanoString multiplex gene expression analysis. Cells were cultured in 12-well plates with TS medium including F4H or ES medium supplemented with LIF. Dissociated cells were collected by trypsinization and centrifugation. Cell pellets were dispensed directly in RNAlater Stabilization Solution (Qiagen) and stored in −80 °C for later use. Cell pellets were lysed in RLT Lysis Plus Buffer using a TissueLyser LT (Qiagen) at 40 Hz for 2 min. Total RNA extraction was performed using RNeasy Plus Micro kit (Qiagen) according to manufacturer's protocol. The custom NanoString CodeSet "Extra" was used. 100 ng of total RNA samples were hybridized at 65 °C for 18 hr and processed with the nCounter Analysis System GEN1 (NanoString Technologies). The reporter counts were processed using nSolver Analysis Software v2.5 (NanoString). Two normalizations were performed to the counts, the first normalization to the generic positive controls, followed by normalization to the reference genes, *Actb* and *Gapdh*. Normalized counts are displayed in a heatmap generated by the nSolver Analysis Software v2.5, using agglomerative clustering.

NanoString probe sequences. Below are the nucleotide sequences of the NanoString probes; first line is the capture probe, second line the reporter probe.

Actb
ACGATGGAGGGGCCGGACTCATCGTACTCCTGCTTGC
GGGTGTAAAACGCAGCTCAGTAACAGTCCGCCTAGAAGCACTTGCGGTGC
Dab2
GTCTCCTCGAGCATCAGGCACATCATCAATACCGATTAGCTTGGC
CCAGCTGCTGCCATTCCCTTGAGTTTCATCATAGAATCCTGACTCATTTT
Dnmt3l
GAGGCAGCGCATACTGCAGGATCCGGTGGAACTGGAACATG
CCATGAATATCCAGAAGAAGGGCCGCTGACTCTCCTGGC
Dppa4

CAAGTCTTTACAGTTGACTGCTGAACTGGTTATGACGCCCGTTGTGCTGG
CACTACAACCCAGGGAAGAGGACATGCATGCGGAGGCTACAGGTATAAGC
Dppa5a
CGCACGGCCCACAGCTCCAGGTTCAGGAAGTTTTAGTAC
CCTGCCAAGGAACCAGACTTCAGGGAAGACGAGATCAAGCTTATCCACCA
Esrrb
TTTCCAGAATGAACCGCTTCATCTTTAGGACATCCTGTCAACCCAAACCC
GAGCAGGTAAAGCCGGAGGACTTGTCATGAAAGTGGCGTGTCCAT
Fgf4
ATTCTGGTAACAAAATTCCAAAGATACAGTCTTGTCCCTGGGCGCAGGAA
ACAGACCGACTCGGTAACAGTGGCAGATACAGAGCAGAAACATCAAACCC
Foxa2
CACAGACAGGTGAGACTGCTCCCTTGAGGCCTGAAG
TCCCTTCCCTATTTAGAATGACAGATCACTGTGGCCCATCTATTTAGGGA
Foxq1
GCTGTCCTTACTCCGAGGTTTAGAGACTTTGAGCGGAAGACAAGCG
TTTTGATTGTTGGGTGAAGTGAGGAGTGGAGTGATAGAAGTTGGTGCAGT
Fst
AGGACTTTGTGATACACTTTCCCTCATAGGCTAATCCAATGGATCTGCCC
CTTGGAATCCCATAGGCATTTTTTCCCGCCGCCACACTGGATATCTTCAC
Fxyd3
TGAGCCCGCCGACTCGGAGGCTGTACCAATCATAGTAGAAAGGATCATTT
GCCACTCATAAGGACTATAATGCCCAGGGCACAGAGAATCCCTGCACAAA
Gapdh
ATACTTGGCAGGTTTCTCCAGGCGGCACGTCAGATCCACGACG
CCTCAGATGCCTGCTTCACCACCTTCTTGATGTCATC
Gata4
AGAGCCAGGTAACTGTCTGACTTAAGAGGGCTTGGCTTGG
GCAAACAGTCTGTATTTTCTAGACAAAGGATCTGTGCTGGAGAAAGTCCC
Gata6
ATCTGGACTGCTGGACAATATCAGACACAAGTGGTATGAGGCCTTCAGAG
GGCACAGAAATCACGCATCGAAGGAATGTTATGTCTGCATTTTTGCTGCC
Krt8
TTCCCATCTCGGGTTTCAATCTTCTTCACAACCACAGCCTTG
CAGTGGCCATTCACTTGGACACGACATCAGAAGACTCGGACACCAGC
Lama1
CATTGGCTAAATCGGCATGGCGGTCATCCTTGATACAGACAGAACTCAGA
CATAACCTTTCCTACATGGACACTGACCTGGCCACTTTC
Lamb1
CCAGGAAGGAATGCGGTCCTGAATGTACTGCCTTTCCACCACAATGAC
AAAAACTCCAAATAAGCCCCTTCAGGCACCCGGACGAACCCAGGTCCTGT
Nanog
CATATTTCACCTGGTGGAGTCACAGAGTAGTTCAGGAATAATTCCAAGGC
GAAGGAACCTGGCTTTGCCCTGACTTTAAGCCCAGATGTTGCGTAAGTCT
Nr0b1
CACTTGAAAAAGAAACTCTTGATGGCCTGGACCGCAGCAGCTGGGAGCAA
CCTTTCAGATAGGCATACTCTTTGGTGTCAATGTTCAGACTCCAG
Pdgfra
TATGGAGTAAGTCGCTCTCACACACTTACCACACCACCATGTTGGGAACA
AAACATGAACAGGGGTATCTGGAAGCCATCTTGTATTGGAAGACCCTTCC
Pou5f1
ACATGGTCTCCAGACTCCACCTCACACGGTTCTCAATG
AGTGATCTGCTGTAGGGAGGGCTTCGGGCACTTCAGAA
Pth1r
CCTGGGCACGGTGCAGCAGGAAAATCTGTTCCTCTTTGGTAAAGACATCG
TGCTGTGTGCAGAACTTCCTTGAGCAGCTTGTCACATTGCG
Sox2
CCCCGCCGCCCTCAGGTTTTCTCTGTACAAAAATAGTCCCCCAAAAAGAA
TGCGTAGTTTTTTTCCTCCAGATCTATACATGGTCCGATTCCCCCGCCCT
Sox7
AGAAATCAGCACACCCCAACACTTTTGTGGACAGACGTTTTAGGTTTCTA
TCATCTTTTGCATCTGTAGATAAGAGTATGCTACAGCTCTGCTCT
Sox17
GGCAGATACTGTTCGAATTCCGTGCGGTCCACCTC
TGTCCCTGGTAGGGAAGACCCATCTCGGGCTTATACACAAAG
Tet3
TTTTCAAAGAGCTGAATGAATGCACCAGGATTTTAGGATGGGCGTGTTTC
GTCAGGCCCAACCTCTCAATGTCACAGAAATTAAAGCACCAACGTTCTCA

Ethics statement. All animal studies were carried out in accordance with the German Animal Welfare Act, European Communities Council Directive 2010/63/EU, and institutional ethical and animal welfare guidelines of the Max Planck Institute of Biophysics and the Max Planck Research Unit for Neurogenetics. All experimental protocols were approved by the Regierungspräsidium Darmstadt and the Veterinäramt of the City of Frankfurt.

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Author Contributions

J.L. designed research, performed experiments, and analyzed data. M.K. and B.Z. performed experiments and analyzed data. P.M. supervised research and wrote the manuscript. All authors contributed to editing the manuscript.

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Part III

PDGFRA is not essential for the derivation and maintenance of mouse extraembryonic endoderm stem cell lines

Stem Cell Reports
Report
OPEN ACCESS

PDGFRA Is Not Essential for the Derivation and Maintenance of Mouse Extraembryonic Endoderm Stem Cell Lines

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SUMMARY

Extraembryonic endoderm stem (XEN) cell lines can be derived and maintained *in vitro* and reflect the primitive endoderm lineage. Platelet-derived growth factor receptor alpha (PDGFRA) is thought to be essential for the derivation and maintenance of mouse XEN cell lines. Here, we have re-evaluated this requirement for PDGFRA. We derived multiple PDGFRA-deficient XEN cell lines from postimplantation and preimplantation embryos of a PDGFRA-GFP knockout strain. We also converted PDGFRA-deficient embryonic stem cell lines into XEN cell lines chemically by transient culturing with retinoic acid and Activin A. We confirmed the XEN profile of our 12 PDGFRA-deficient cell lines by immunofluorescence with various markers, by NanoString gene expression analyses, and by their contribution to the extraembryonic endoderm of chimeric embryos produced by injecting these cells into blastocysts. Thus, PDGFRA is not essential for the derivation and maintenance of XEN cell lines.

INTRODUCTION

The mouse preimplantation embryo comprises three cell lineages: trophectoderm, epiblast, and primitive endoderm (PrE) (Artus and Hadjantonakis, 2012). Extraembryonic endoderm stem (XEN) cell lines can be derived and maintained *in vitro* (Niakan et al., 2013), and reflect the PrE lineage. There are four methods to derive mouse XEN cell lines. First, XEN cell lines can be derived directly from blastocysts (Kunath et al., 2005). Second, XEN cell lines can be converted from embryonic stem cells (ESCs) by forced expression of XEN-specific genes such as *Gata6* (Wamaitha et al., 2015), *Gata4* (Fujikura et al., 2002), or *Sox17* (McDonald et al., 2014), or chemically by transient culturing with retinoic acid (RA) and Activin A (Cho et al., 2012). Third, XEN cell lines can be induced from fibroblasts by overexpression of the classical OSKM factors (Parenti et al., 2016). Fourth, we have reported the efficient derivation of XEN cell lines from postimplantation embryos (Lin et al., 2016).

The model of sequential expression of PrE lineage-specific genes is *Gata6* > *Pdgfra* > *Sox17* > *Gata4* > *Sox7* (Artus et al., 2010, 2011). Cells that express *Pdgfra* can be visualized in a gene-targeted knockout mouse strain in which a fusion protein of human histone H2B with GFP is expressed from the *Pdgfra* locus (Hamilton et al., 2003). In this strain, which we refer to as platelet-derived growth factor receptor alpha (PDGFRA)-GFP, the GFP reporter is coexpressed with endogenous PDGFRA protein and with PrE markers GATA6, GATA4, and DAB2 in preimplantation embryos (Plusa et al., 2008). GFP colocalizes in the same cells with PrE markers GATA6 and GATA4 in blastocysts cultured *in vitro*, and is expressed in the visceral and parietal endoderm of postimplantation embryos (Artus et al., 2010). GFP also colocalizes in the same cells with PrE markers SOX17 and SOX7 (Artus et al., 2011). XEN cell lines derived from PDGFRA-GFP heterozygous blastocysts display the intrinsic fluorescence of GFP (Artus et al., 2010). Thus, in this strain GFP serves as a robust live marker for PrE and its extraembryonic endoderm derivatives, and can be applied in the context of XEN cell line derivation.

Because the PDGFRA-GFP mutation represents a knock out of the *Pdgfra* gene, the requirement for PDGFRA can be evaluated in embryos and cells that are homozygous and thus PDGFRA deficient. Out of 74 GFP+ blastocysts from PDGFRA-GFP heterozygous intercrosses, 20 heterozygous, but no homozygous XEN cell lines were isolated (Artus et al., 2010). Likewise, cXEN cells could not be converted chemically from PDGFRA-GFP homozygous ESCs (Cho et al., 2012). Here we have re-evaluated the requirement for PDGFRA in the derivation and maintenance of XEN cell lines.

RESULTS

Post-XEN Cell Lines from PDGFRA-Deficient Postimplantation Embryos

We collected embryonic day 6.5 (E6.5) embryos from PDGFRA-GFP heterozygous intercrosses, and removed as much of the ectoplacental cone from the embryos as possible. We placed each embryo in a well of 4-well dish, coated with gelatin and covered with mouse embryonic fibroblasts (MEF). We cultured the embryos in standard trophoblast stem (TS) cell medium including 25 ng/mL FGF4 and 1 μg/mL heparin (F4H) (Figure 1A). After 5 days, the embryos formed a large outgrowth. We then used TrypLE Express to disaggregate the outgrowths, and

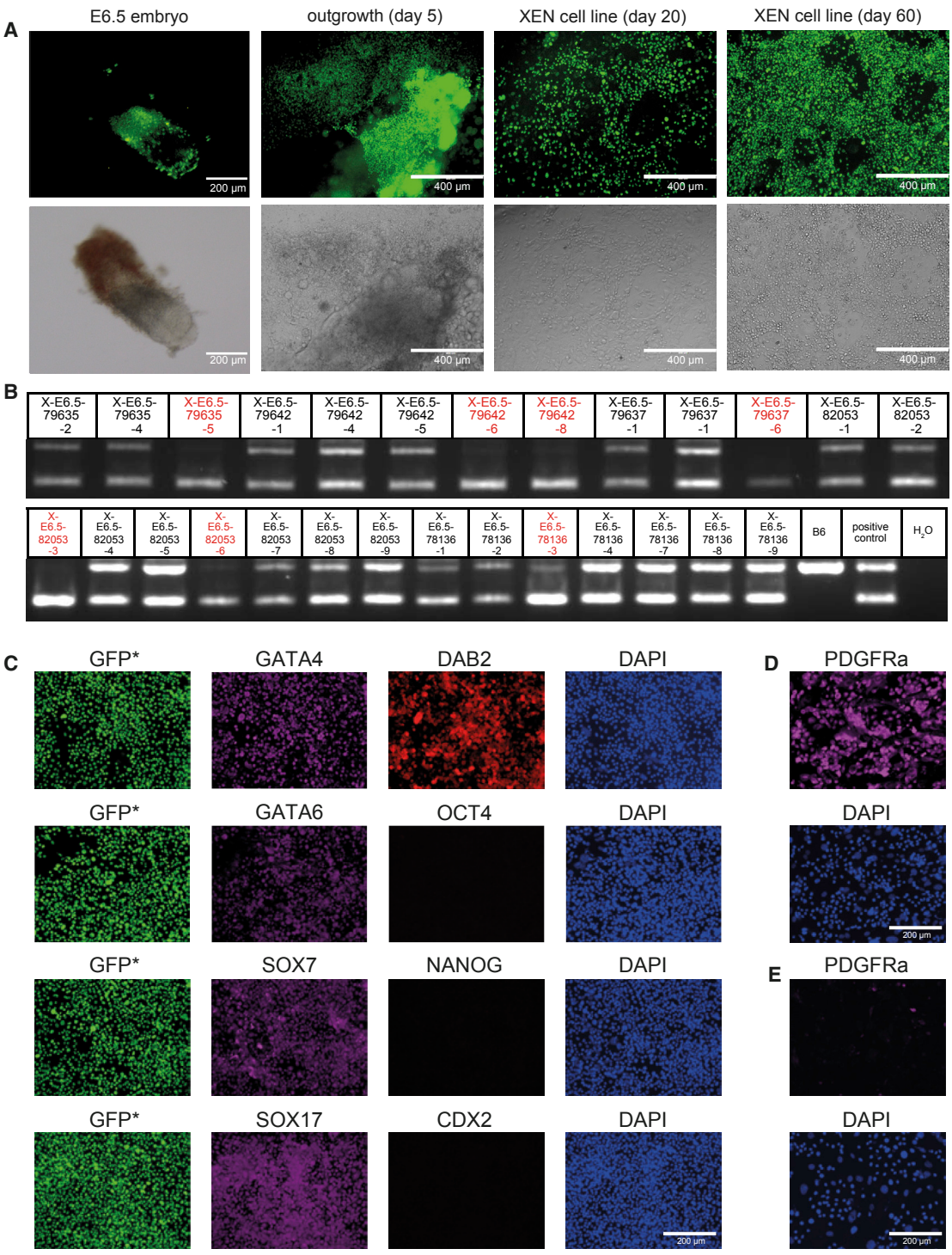

Figure 1. Post-XEN Cell Lines Derived from PDGFRA-Deficient Postimplantation Embryos

(A) Post-XEN cell line X-E6.5-79642-8 derived from a PDGFRA-deficient E6.5 embryo.

(B) Genotyping results. Positive control: genomic DNA from the tail of a PDGFRA-GFP heterozygous mouse. B6: genomic DNA from the tail of a C57BL/6J mouse. Red, PDGFRA-GFP homozygous XEN cell lines.

(legend continued on next page)

passaged them into a well of a 4-well dish. When cells reached 70%–80% confluency, they were passaged into a well of a 12-well dish until a stable cell line was obtained, which was then passaged routinely in a well of a 6-well dish. We thus derived 27 post-XEN cell lines from 31 GFP+ embryos from PDGFRA-GFP heterozygous intercrosses. Genotyping by PCR of genomic DNA indicated that seven post-XEN cell lines are homozygous for the PDGFRA-GFP knockout mutation (Figure 1B), and are thus PDGFRA-deficient. Five of the seven PDGFRA-deficient post-XEN cell lines were maintained for >60 days, and resemble conventional XEN cell lines. Immunofluorescence analyses indicated that PDGFRA-deficient post-XEN cell lines are positive for XEN cell markers DAB2, GATA4, GATA6, SOX7, and SOX17, but negative for ESC marker NANOG and OCT4, and negative for TS cell marker CDX2 (Figure 1C). PDGFRA-GFP heterozygous cell line X-E6.5-79642-1 is immunoreactive for PDGFRA, demonstrating that this antibody works (Figure 1D). By contrast, PDGFRA-GFP homozygous cell line X-E6.5-79642-8 is not immunoreactive for PDGFRA, consistent with the knockout design of the targeted mutation (Figure 1E).

Derivation of a Pre-XEN Cell Line from a PDGFRA-Deficient Blastocyst

In a first set of experiments, we collected E1.5–E2.5 embryos from PDGFRA-GFP heterozygous intercrosses, and cultured them in KSOM medium to the blastocyst stage. We then removed the zona pellucida using acid Tyrode's solution. We transferred each of 24 GFP+ blastocysts into a well of a 4-well dish, coated with 0.1% gelatin and covered with MEF, and cultured them in ES medium with leukemia inhibitory factor (LIF). An example is shown in Figure 2A: an outgrowth started to form on day 1. On day 4 the outgrowth was larger and still contained GFP+ cells. On day 5 we disaggregated the outgrowth and passaged cells into a well of a 4-well dish, coated with gelatin, and covered with MEF. Our strategy was to change the medium only every 2–3 days, and to passage cells only every week or two. We reasoned that more frequent cell passaging would dilute the XEN cells because ESCs grow much faster than XEN cells in these mixed cultures. Large, ES-like colonies developed with time. On day 12 in this example (and in other cases between days 10 and 15), we removed as many of the ES-like colonies as possible using two needles and a pipette but kept XEN-like colonies. From some preimplantation embryos, we derived a pre-XEN cell line, but in other cases the ESCs continued to dominate and we ended up deriving an ESC line. In the example of cell line ES-111, on day 35 we observed large XEN-like colonies with strong GFP expression, and ES-like colonies surrounded by GFP+ cells (Figure 2A). After passaging on day 35, the ESCs overtook the GFP+ cells, and it became no longer possible to remove ES-like colonies. After 60 days we derived six pre-XEN cell lines and three ESC lines (which contained a small fraction of XEN-like cells) from the 24 GFP+ blastocysts. The pre-XEN cell lines were genotyped as PDGFRA-GFP heterozygous, and the ESC lines including ES-111 as PDGFRA-GFP homozygous (Figure 2C).

In a second set of experiments, we isolated by immunosurgery (Lin et al., 2011) the inner cell mass (ICM) from six GFP+ blastocysts from a PDGFRA-GFP heterozygous intercross. We transferred each ICM into a well of a 4-well dish (Figure 2B), in ES medium with LIF and without 2i. On day 2 the ICMs attached to the dish and formed an outgrowth. We changed the medium every 2–3 days without passaging the cells. On day 12, large colonies with two distinct phenotypes were present: XEN-like GFP+ colonies and ES-like GFP− colonies. We picked the XEN-like colonies, and passaged the cells. After several days ES-like colonies appeared again. We removed ES-like colonies as much as possible, and picked XEN-like cell colonies on day 41. We thus derived four pre-XEN cell lines from six GFP+ ICMs. Three pre-XEN cell lines were genotyped as PDGFRA-GFP heterozygous, and X-ICM-97025-4 as PDGFRA-GFP homozygous (Figure 2C). Immunofluorescence analysis indicated that X-ICM-97025-4 is positive for XEN cell markers DAB2, GATA4, GATA6, and SOX17, but negative for ESC marker OCT4, and negative for TS cell marker CDX2 (Figure 2D). PDGFRA-GFP heterozygous cell line X116 is immunoreactive for PDGFRA (Figure 2E), and X-ICM-97025-4 is not (Figure 2F).

Chemical Conversion of PDGFRA-Deficient ESC Lines into cXEN Cells

Using ES medium with LIF and 2i (PD0325901 and CHIR99021), we derived one PDGFRA-GFP heterozygous ESC line (ESC-18) and three PDGFRA-GFP homozygous ESC lines (ESC-22, ESC-23, ESC-24) from eight blastocysts of a PDGFRA-GFP heterozygous intercross. We noticed that, in these ESC lines, sparse GFP+ cells surrounded rare ESC colonies (ESCs typically do not express PDGRFA and are thus GFP−). The occurrence of these cells is in agreement with observations that SOX17 is expressed in a subset of cells on the outside of otherwise undifferentiated ESC

(C) PDGFRA-deficient post-XEN cell line X-E6.5-79642-8. First column, PDGFRA-GFP*: intrinsic green fluorescence of GFP expressed from the gene-targeted *Pdgfra* locus. Second and third columns: immunofluorescence for GATA4, GATA6, SOX7, SOX17, DAB2, OCT4, NANOG, and CDX2. Fourth column: DAPI nuclear strain.
(D and E) X-E6.5-79642-1 is immunoreactive for PDGFRA (D), and X-E6.5-79642-8 is negative (E).

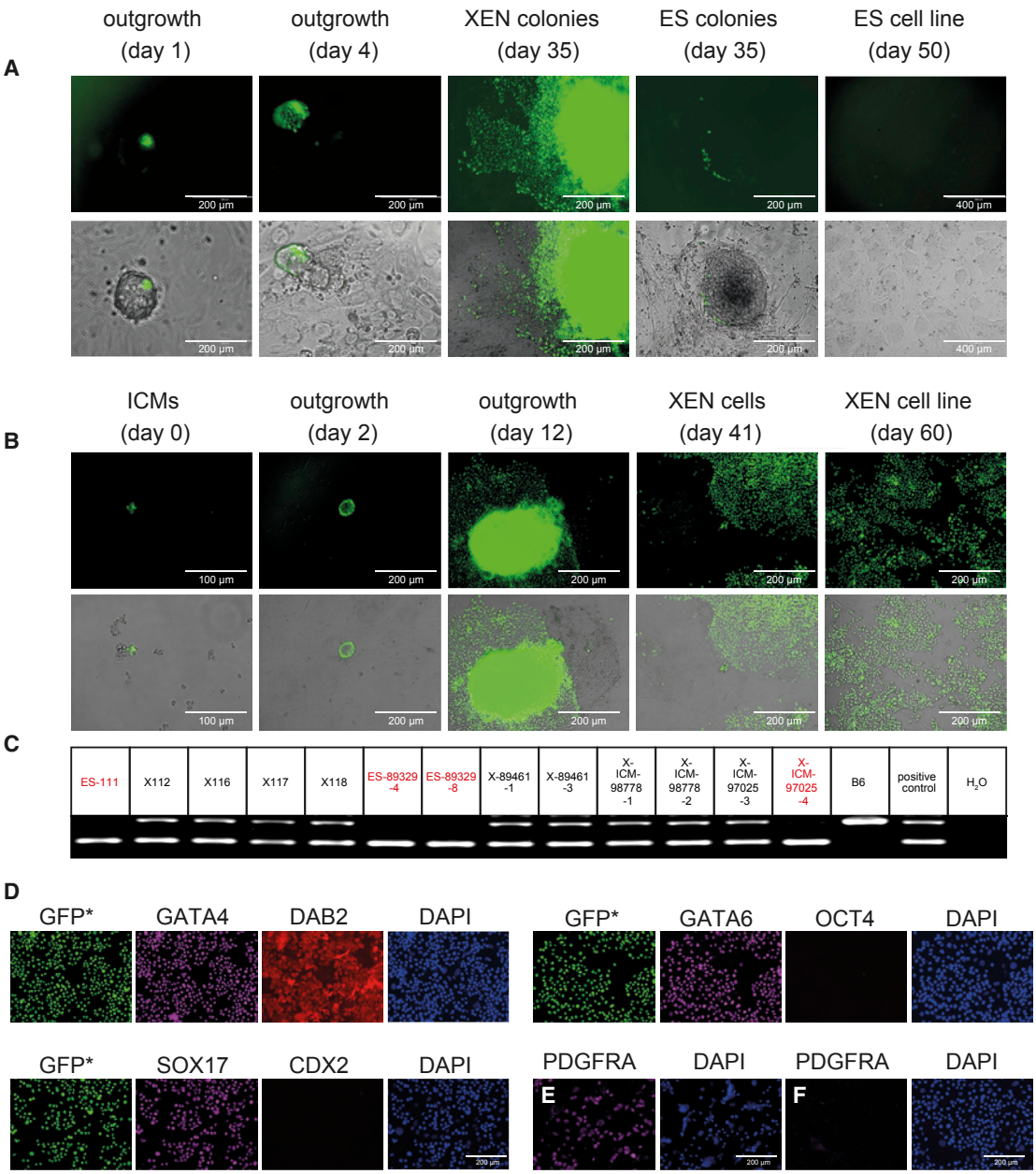

Figure 2. ES and Pre-XEN Cell Lines Derived from PDGFRA-Deficient Blastocysts

(A) ESC line ES-111 derived from a PDGFRA-deficient blastocyst. GFP+ cells are rare on day 50.

(B) Pre-XEN cell line X-ICM-97025-4 derived from a PDGFRA-deficient ICM.

(C) Genotyping results. Positive control: genomic DNA from the tail of a PDGFRA-GFP heterozygous mouse. Red: PDGFRA-GFP homozygous cell lines.

(D) X-ICM-97025-4. First column, PDGFRA-GFP*: intrinsic green fluorescence of GFP. Second and third columns: immunofluorescence for GATA4, GATA6, SOX17, DAB2, OCT4, and CDX2. Fourth column: DAPI nuclear stain (blue).

(E and F) X116 is immunoreactive for PDGFRA (E), and X-ICM-97025-4 is immunonegative (F).

colonies (Niakan et al., 2010), that ESCs cultured in LIF and 2i contain a few cells expressing GATA6 (Morgani et al., 2013), and that PDGFRA-GFP heterozygous and homozygous ESCs contain a fraction of GFP+ cells (Lo Nigro et al., 2017). It thus appears that some ESCs convert spontaneously into XEN or XEN-like cells.

A low dose of RA and Activin A promotes the chemical conversion of ESCs into XEN cells (so-called cXEN cells),

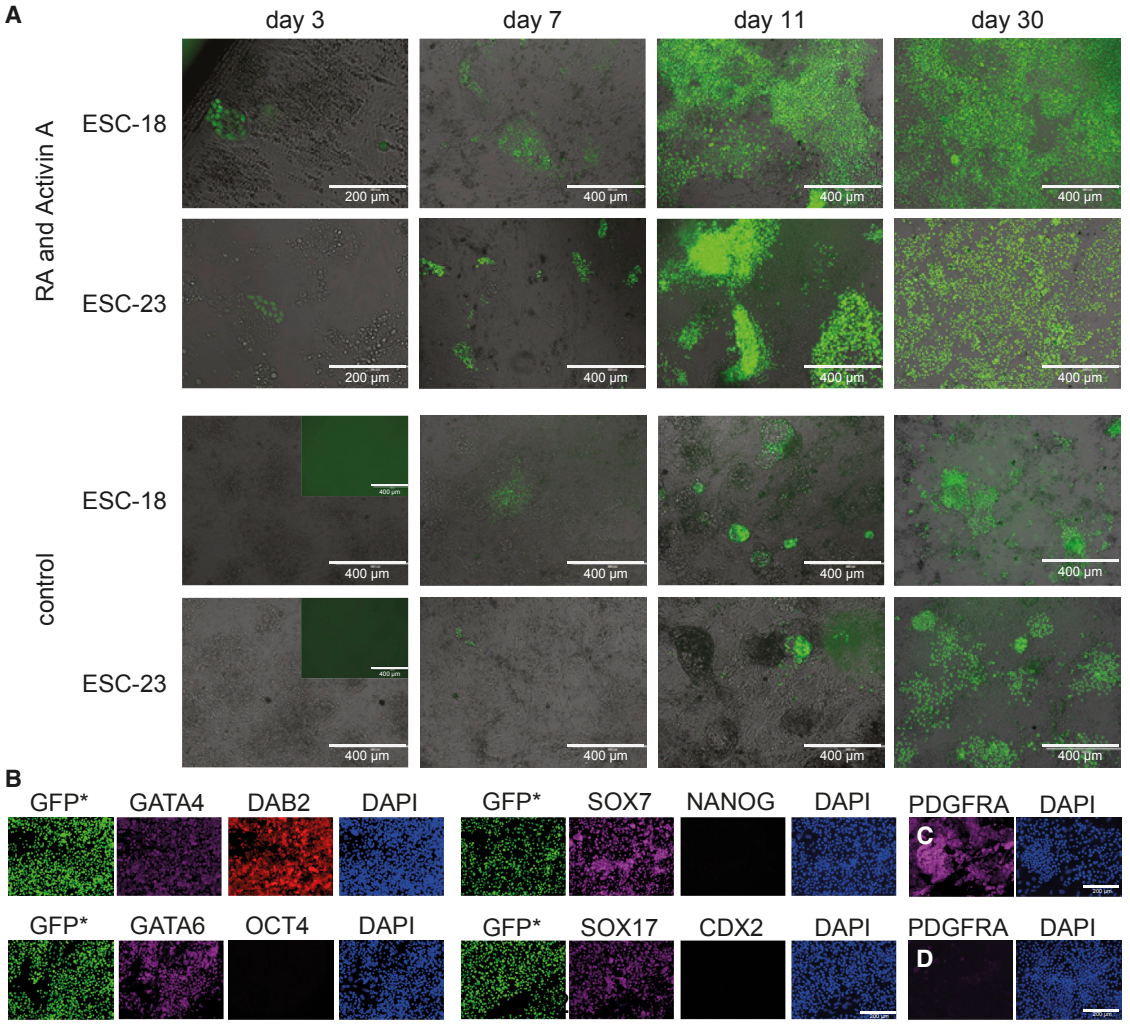

Figure 3. cXEN Cell Lines Converted Chemically from ESC Lines

(A) Conversion of ESC-18 (PDGFRA-GFP heterozygous) and ESC-23 (PDGFRA-deficient) into cXEN cells in TS medium with F4H, 0.01 µM RA, and 10 ng/mL Activin A, or F4H as control. Insets for ESC-18 and ESC-23 in control condition show that there are no GFP+ cells on day 3.

(B) Immunofluorescence on PDGFRA-deficient cXEN-23 cells converted from ESC-23. First column, PDGFRA-GFP*: intrinsic green fluorescence of GFP. Second and third columns: immunofluorescence for GATA4, GATA6, SOX7, SOX17, DAB2, OCT4, NANOG, and CDX2. Fourth column, DAPI.

(C and D) cXEN-18 is immunoreactive for PDGFRA (C), and cXEN-23 is not (D).

but failed to convert PDGFRA-deficient ESCs into cXEN cells (Cho et al., 2012). We followed the cXEN conversion protocol of Cho et al. (2012). We cultured ESC-18 (PDGFRA-GFP heterozygous) and ESC-23 (PDGFRA-GFP homozygous) for 48 hr in standard TS cell medium with F4H, to which 0.01 µM RA and 10 ng/mL Activin A were added; in the control condition, the medium was TS cell medium with F4H. Thereafter all cells were cultured in standard TS cell medium with F4H. XEN-like colonies with GFP expression accumulated on days 7 and 11 (Figure 3A). On day 11 we found that the fraction of GFP+ cells in the conversion treatment is much higher than in the control condition: ESC-18 cells, 77% versus 4%; ESC-23 cells, 23% versus 1.3%. As the cultures grew confluent, a fraction of the GFP+ cells did not adhere tightly to the dishes and were easier to lose during medium changes. It appears that, whereas colonies of ES-like cells and differentiating ESCs adhered tightly to the dishes, XEN-like cells became sorted to the outside of these colonies and then were excluded from the colonies. We therefore enriched systematically for GFP+ cells when a medium change was due, by spinning down the suspended cells and transferring them into a new dish coated with gelatin and covered with MEF. We thus converted

cXEN cell lines from ESC-18 after ~21 days and from ESC-23 after ~30 days (Figure 3A). The PDGFRA-deficient cXEN cell line that we converted from ESC-23 (called cXEN-23) was maintained for >60 days, and retained GFP expression and the XEN cell phenotype in culture. Next we applied this protocol to convert PDGFRA-deficient ESC lines ESC-22 and ESC-24 cells into cXEN cell lines. After ~30 days we obtained a stable cXEN cell line from each ESC line, called cXEN-22 and cXEN-24. Finally, after ~21 days we converted ES-111, a PDGFRA-deficient ESC line that we had derived in medium without 2i, into cXEN-111. We cultured cXEN-111 cells in standard TS cell medium for >120 days, and they retained GFP expression and a XEN-like phenotype (data not shown). Immunofluorescence analysis indicated that the four PDGFRA-deficient cXEN lines are positive for XEN cell markers DAB2, GATA4, GATA6, SOX7, and SOX17, but negative for ESC markers OCT4 and NANOG, and negative for TS cell marker CDX2; images are shown for cXEN-23 (Figure 3B). PDGFRA-GFP heterozygous cell line cXEN-18 is immunoreactive for PDGFRA (Figure 3C), but PDGFRA-deficient cell line cXEN-23 is not (Figure 3D).

NanoString Gene Expression Analyses of XEN Cell Lines and ESC Lines

Next we applied the NanoString multiplex platform (Khan et al., 2011) to compare patterns of gene expression in PDGFRA-GFP homozygous and heterozygous ES and XEN cell lines. All XEN cell lines had high levels of expression of XEN cell-specific genes such as *Gata4*, *Gata6*, *Sox17*, *Sox7*, and *Dab2*, versus low levels of expression or no expression of ESC-specific genes such as *Sox2*, *Pou5f1*/*Oct4*, *Nanog*, and *Zfp42*/*Rex1* (Figure 4A). In PDGFRA-GFP homozygous XEN cell lines, *Pdgfra* expression is, as expected, absent or highly reduced; the residual RNA is from the remaining MEF. There are no differentially expressed genes other than *Pdgfra* itself.

PDGFRA-Deficient XEN Cells Contribute to the Parietal Endoderm

A cell proliferation assay revealed no difference in the growth rate of PDGRA-GFP heterozygous and homozygous cell lines (Figure 4B).

To test their *in vivo* potential, we injected cells of four post-XEN cell lines (X-E6.5-79642-8, X-E6.5-79637-6, X-E6.5-82053-3, and X-E6.5-82053-6), one PDGFRA-deficient pre-XEN cell line (X-ICM-97025-4), and one PDGFRA-deficient cXEN cell line (cXEN-111) into blastocysts of C57BL/6J or CD1 origin, and transferred the injected blastocysts into pseudopregnant recipients. We transferred 33 blastocysts injected with X-E6.5-79642-8, identified 27 implantation embryos at E7.0–E8.0, and recovered 18 embryos, among which there were two chimeras. For X-E6.5-79637-6, the numbers were 43, 32, 26, and 2; for X-E6.5-82053-3, the numbers were 17, 17, 10, and 0; for X-E6.5-82053-6, the numbers were 10, 4, 4, and 0; for X-ICM-97025-4, it was 33, 20, 15, and 3. Finally, for cXEN-111, the results were 43, 40, 13, and 1. In total, we transferred 179 blastocysts injected with PDGFRA-deficient XEN cells, we identified 140 implantation sites at E7.0–E8.0, and we recovered 86 embryos, 8 of which (9%) had GFP+ cells contributing to their parietal endoderm (Figures 4C–4H).

DISCUSSION

We have derived a dozen PDGFRA-deficient XEN cell lines: seven post-XEN cell lines, one pre-XEN cell line, and four cXEN cell lines.

Why are PDGFRA-deficient XEN cell lines easier to derive from postimplantation embryos than from preimplantation embryos? First, the missing PDGFRA signal reduces the number of PrE cells in blastocysts (Artus et al., 2013). These remaining PrE cells still have the ability to support fetal development to the prenatal stage (Hamilton et al., 2003); we identified six homozygous fetuses among a total of 28 fetuses (=22%) from PDGFRA-GFP heterozygous intercrosses (data not shown). We observed that GFP+ cells could be maintained in culture and grew slowly to form large colonies. But in the mixed ES-XEN cultures that we derived from blastocysts, ESCs grew much faster than XEN cells, and ESCs dominated after several passages. Second, when derivation of XEN cell lines is attempted from postimplantation embryos, the pluripotent epiblast cells have already differentiated and cells may convert spontaneously to XEN cells. XEN cells could be derived from the extraembryonic endoderm or converted from the epiblast of the postimplantation embryo, and dominated the culture with time. Third, the extraembryonic endoderm has more cells than the PrE (Morris et al., 2010; Snow, 1977).

Why were we able to chemically convert PDGFRA-deficient ESC lines into cXEN lines, whereas Cho et al., 2012 were not? First, we applied infrequent cell passaging. The conventional method is to passage cells frequently (Niakan et al., 2013). We observed that PDGFRA-deficient ESCs are more difficult to convert than PDGFRA-GFP heterozygous ESCs in TS cell medium with F4H. Second, we collected cells suspended in the culture medium and spun down the medium to enrich for GFP+ cells (XEN-like) after plating in new dishes. We found that XEN cells cultured in TS cell medium are easier to collect in suspension than in ES medium, when colonies become crowded. The conventional method to change medium and passage cells entails

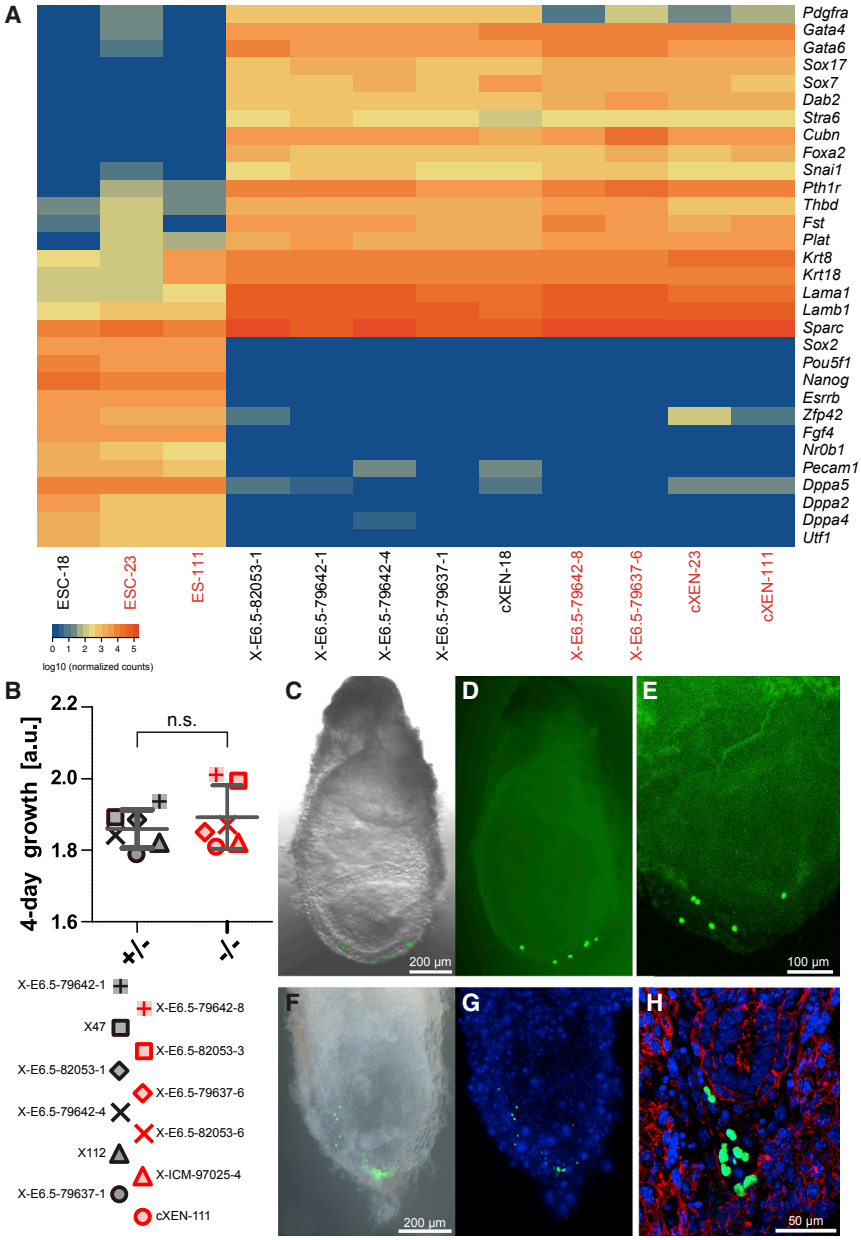

Figure 4. Cell Growth, NanoString Gene Expression Analysis, and Chimeric Embryos

(A) Heatmap NanoString analysis of one PDGFRA-GFP heterozygous ESC line (ESC-18), two PDGFRA-deficient ESC lines (ESC-23, ESC-111), five PDGFRA-GFP heterozygous XEN cell lines (X-E6.5-82053-1, X-E6.5-79642-1, X-E6.5-79642-4, X-E6.5-79637-1, and cXEN-18), and four PDGFRA-deficient XEN cell lines (X-E6.5-79642-8, X-E6.5-79637-6, cXEN-23, and cXEN-111). The PDGFRA-deficient cell lines are indicated in red. Heatmap colors correspond to $\log_{10}$ values of normalized counts as indicated in the color key, from dark blue (low) to dark orange (high).

(B) Cell proliferation: a.u. (arbitrary units), difference between days 4 and 1. n.s., not significant (t test). Line at mean, error bars at SD. Six cell lines were used for each of the two genotypes, representing six biological replicates per genotype. Three technical replicates per cell line were seeded and measured on a daily basis for 4 days.

(C–E) PDGFRA-deficient post-XEN cell line X-E6.5-79642-8. A whole mount of an E7.5 chimeric embryo was imaged in bright field and fluorescence (C) and in fluorescence (D), using a Nikon SMZ25 stereofluorescence microscope. The same embryo was imaged using a Zeiss LSM 710 confocal microscope (E).

(F–H) PDGFRA-deficient pre-XEN cell line X-ICM-97025-4. A whole mount of an E7.5 chimeric embryo was imaged in bright field and fluorescence (F) using a Nikon SMZ25, and in fluorescence alone (G), with DAPI (blue) and GFP (green), using a Zeiss LSM 710. A section of the decidua of another E7.5 embryo, showing the merged image of fluorescence from DAPI, GFP, and F-actin (red), was imaged using a Zeiss LSM 710 (H).

removing the culture medium, which would also remove the suspended (XEN-like) cells.

The PDGFRA-deficient XEN cell lines are healthy, grow as well as wild-type and PDGFRA-GFP heterozygous XEN cell lines, and differ thus far only in *Pdgfra* expression from PDGFRA-GFP heterozygous XEN cell lines. The rate of chimeras among recovered embryos, however, is lower (9%) than we obtained with PDGFRA-GFP heterozygous and other genetically marked pre- and post-XEN cell lines (35%–39%, Lin et al., 2016). Further experiments, such as RNA sequencing, may reveal differences in gene expression between PDGFRA-deficient and PDGFRA-GFP heterozygous XEN cell lines. Some of these differences may explain the lower rate of chimera formation.

EXPERIMENTAL PROCEDURES

Mice

The PDGFRA-GFP strain was B6.129S4-Pdgfra < tm11(EGFP) Sor>/J (The Jackson Laboratory, no. 7669). MEF were prepared from Tg(DR4)1Jae (The Jackson Laboratory, no. 3208). Mouse experiments were performed in accordance with the German Animal Welfare Act, the European Communities Council Directive 2010/63/EU, and the institutional ethical and animal

welfare guidelines of the Max Planck Research Unit for Neurogenetics.

TS Cell Medium

Advanced RPMI-1640 (Gibco, no. 12633-012) was supplemented with 20% (vol/vol) fetal bovine serum (FBS) (HyClone, no. SH30071.03), 2 mM GlutaMAX Supplement (Gibco, no. 35050), 1% penicillin/streptomycin (Specialty Media, no. TMS-AB2-C), 0.1 mM β-mercaptoethanol (Gibco, no. 21985-023), and 1 mM sodium pyruvate (Gibco, no. 11360-039); and with F4H, which consists of 25 ng/mL FGF4 (PeproTech, no. 100-31) and 1 μg/mL heparin (Sigma, no. H3149).

ESC Medium

DMEM (Specialty Media, no. SLM-220) was supplemented with 15% FBS (HyClone, no. SH30071.03), 2 mM GlutaMAX Supplement, 1% penicillin/streptomycin, 1% β-mercaptoethanol (Specialty Media, no. ES-007-E), 0.1 mM nonessential amino acids (Gibco, no. 11140-035), 1 mM sodium pyruvate, and 1,000 IU/mL LIF (Millipore, no. ESG1107).

cXEN Cell Conversion from ESCs with RA and Activin A

The chemical conversion was performed as described previously (Cho et al., 2012), with modifications. In the XEN culture medium, we increased FBS from 13% to 20%, and added 1 mM sodium pyruvate. ESCs were cultured in ES medium with LIF until they reached 70%–80% confluency, then in standard TS medium with F4H. After 24 hr, the medium was changed to TS medium with F4H, 0.01 μM all-*trans* RA (Sigma, no. R2625) and 10 ng/mL Activin A (R&D Systems, no. 338-AC-010). After 48 hr, we changed the culture medium to TS medium with F4H. Cells were maintained hereafter in standard TS medium with F4H. After 24 hr, cells were dissociated with TrypLE Express and plated at a 1:2 dilution in a dish coated with gelatin and with or without MEF. Around day 15, a fraction of GFP+ cells did not adhere tightly to the dishes. We collected the culture medium into a 2.0 mL Eppendorf tube, centrifuged the tube for 30 s in a Sprout minicentrifuge, and removed the supernatant. We washed the dishes twice with calcium and magnesium-free PBS, transferred the PBS with suspension cells to the tube, centrifuged the tube, and removed the supernatant. Finally we added fresh medium to the tube, and transferred the medium including the pelleted cells back into new dishes coated with gelatin and covered with MEF. We applied this method to collect GFP cells every day while changing medium.

Immunofluorescence and Imaging

Cell lines were cultured in 4- or 24-well dishes. Cells were fixed in 4% paraformaldehyde at 4°C overnight or room temperature for 30 min, permeabilized with 0.1% Triton X-100 in PBS (PBST) for 30 min and blocked with 5% normal donkey serum (Jackson ImmunoResearch Laboratories, no. 017-000-121) diluted in PBST (blocking solution) for 1 hr. Primary antibodies were diluted at 1:50–1:500 in blocking solution and samples incubated at 4°C rotating overnight. After three 10-min washes in PBST, samples were incubated for 1–1.5 hr at room temperature in a 1:500 dilution of secondary antibody in blocking solution, then washed and covered with PBST containing DAPI. Primary antibodies from Santa Cruz Biotechnology were against GATA4 (no. SC-1237), DAB2 (no. SC-13982), OCT3-4 (no. SC-5279), NANOG (no. SC-376915), and CDX2 (no. SC-166830). Primary antibodies from R&D Systems were against GATA6 (no. AF1700), SOX7 (no. AF2766), SOX17 (no. AF1924), and PDGFRA (no. AF1062). Secondary antibodies from Jackson ImmunoResearch Laboratories were Cy5 AffiniPure Donkey anti-Goat IgG (H+L) (no. 705-175-147). Secondary antibodies from Invitrogen were Donkey anti-Rabbit IgG (H+L) with Alexa Fluor 546 (no. A10040), and Donkey anti-Mouse IgG with Alexa Fluor 546 (no. A10036).

Cell Proliferation Assay

Cells were cultured in 12-well dishes with MEF; pre-XEN cell lines in ES medium with LIF, and post-XEN and cXEN cell lines in TS medium with F4H.

XEN cell lines were treated by TrypLE Express for 5 min, disaggregated, spun down, resuspended in 100 μL TS medium and 900 μL PBS, and put through a 40 μm filter to collect single cells. Cells were counted in a Countess Cell Counting Chamber Slides (Invitrogen, no. C10228). We plated 5,000 cells in triplicate into wells of 96-well dishes, coated with gelatin, and covered by MEF. Cells were cultured in ES medium with LIF, and the medium was changed on the second and third day. To count cells, the medium was removed, 100 μL fresh ES medium with LIF was added, followed by 10 μL Cell Counting Kit-8 (Sigma, no. 96992). Cells were cultured for another 4 hr, and absorbance was measured at 485 nm with a Tecan Infinite 200 PRO plate reader.

NanoString Multiplex Gene Expression Analysis

Cells were collected by trypsinization and centrifugation. Cell pellets were dispensed in RNAlater Stabilization Solution (QIAGEN) and stored at −80°C for later use. Cell pellets were lysed in RLT Lysis Plus Buffer using a TissueLyser LT (QIAGEN) at 40 Hz for 2 min. Extraction of total RNA was performed using the RNeasy Plus Micro kit (QIAGEN). The custom NanoString CodeSet "Extra" was used; sequences of relevant capture and reporter probes are in the Supplementary Information. An aliquot of 100 ng was hybridized at 65°C for 18 hr and processed with nCounter (NanoString Technologies). Background subtraction was performed using the maximum count of the negative control. A two-step normalization was done: (1) the geometric mean of positive controls was used as the normalization factor across samples, and (2) the geometric mean of *Actb* and *Gapdh* counts was used as biological reference normalization factor. Heatmap was generated using heatmap.2 function in R package gplots.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.08.005.

AUTHOR CONTRIBUTIONS

J.L., M.K., and B.Z. performed the experiments. P.M. managed the project and wrote the paper.

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Part IV

Exclusive transmission of the embryonic stem cell-derived genome through the mouse germline

 genesis 54:326–333 (2016)

RESEARCH ARTICLE

Exclusive Transmission of the Embryonic Stem Cell-Derived Genome Through the Mouse Germline

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Summary: Gene targeting in embryonic stem (ES) cells remains best practice for introducing complex mutations into the mouse germline. One aspect in this multistep process that has not been streamlined with regard to the logistics and ethics of mouse breeding is the efficiency of germline transmission: the transmission of the ES cell-derived genome through the germline of chimeras to their offspring. A method whereby male chimeras transmit exclusively the genome of the injected ES cells to their offspring has been developed. The new technology, referred to as goGermline, entails injecting ES cells into blastocysts produced by superovulated homozygous *Tsc22d3* floxed females mated with homozygous *ROSA26-Cre* males. This cross produces males that are sterile due to a complete cell-autonomous defect in spermatogenesis. The resulting male chimeras can be sterile but when fertile, they transmit the ES cell-derived genome to 100% of their offspring. The method was validated extensively and in two laboratories for gene-targeted ES clones that were derived from the commonly used parental ES cell lines Bruce4, E14, and JM8A3. The complete elimination of the collateral birth of undesired, non-ES cell-derived offspring in goGermline technology fulfills the reduction imperative of the 3R principle of humane experimental technique with animals. genesis 54:326–333, 2016. © 2016 The Authors. Genesis Published by Wiley Periodicals, Inc.

Key words: embryonic stem cell; sterility; Tsc22d3; Gilz

INTRODUCTION

Gene targeting in mice via homologous recombination in embryonic stem (ES) cells has been extraordinarily informative in all fields of biomedical research (Capecchi, 2008; Evans, 2008; Smithies, 2008). The novel technology of CRISPR-Cas9 can introduce gene edits directly into the genome of the mouse zygote and thereby obviates the intermediary vehicle of ES cells (Yang *et al*., 2014). But for complex genetic modifications such as floxed alleles, bicistronic mutations, and knockins of large DNA segments, gene targeting in ES cells, as we know it since the late 1980s, remains best practice.

One aspect in this long multistep process that has not been streamlined with regard to the logistics and ethics of mouse breeding is the efficiency or rate of germline transmission (GLT): the transmission of the ES cell-derived genome through the germline of male chimeras to their offspring (Bradley *et al*., 1984). Such chimeras are referred to as germline chimeras. It is not possible to screen for germline transmission other than by the biological test of breeding the chimeras, but numerous undesired, non-ES cell-derived offspring are hereby born collaterally. Male chimeras are typically selected for breeding on the basis of high somatic ES cell contribution as assessed visually by coat color chimerism, and germline offspring are identified by coat color. Among the ES cell-derived offspring, 50% inherit the targeted mutation, if it is present in a heterozygous state in the injected ES cells. But it remains difficult to predict the rate of GLT for an individual chimera or for

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a set of chimeras generated with a particular gene-targeted ES cell clone. Overbreeding may be the result, in particular when there is urgency in establishing a novel gene-targeted strain: too many chimeras are set up for breeding, too many offspring are sired, too many non-ES cell derived offspring are born and these are of no further use and are typically culled. A substantial fraction of injected ES cell clones result in chimeras that sire only undesired, non-ES cell-derived offspring.

Over the years attempts have been made to improve the rate of GLT, but none of these methods have become standard practice. Chimeras with 100% GLT were already reported in 1993 by aggregating ES cells with tetraploid blastocysts (Nagy *et al*., 1993) but the birth rate is low. The Perfect Host approach (Taft *et al*., 2013) promised to improve GLT rates by generating male chimeras with diphtheria-toxin mediated ablation of the host germline. But this approach, as it was described, is imperfect in that it disregards the practical advantages of coat color differences between strains used to derive the ES cell lines and strains used to produce blastocysts. Moreover the Perfect Host approach was tested out only for 11 gene-targeted ES cell clones and in only one laboratory. There is no report in the literature that has made since then use of this approach.

Here we report the development of a new technology, called goGermline, that affords 100% GLT by male chimeras. The technology is based on the unexpected observation that males with a mutation in the gene *Tsc22d3*, also called *Gilz*, are completely sterile due to a cell-autonomous defect in spermatogenesis, and relies further on the convenient location of *Tsc22d3* on the X chromosome. When colonizing the germline of hemizygous *Tsc22d3* knockout males, cells that descended from the injected ES cells have no competition from cells that descended from the host embryo. The male chimeras can be sterile but, when fertile, they transmit the ES cell-derived genome to 100% of their offspring. Fertile chimeras thus behave genetically as heterozygotes. The technology is so efficient that in our current standard operating protocol, a project is considered successful as soon as females mated with chimeras are observed to be pregnant; no further injections need to be scheduled, and these or additional chimeras need not be bred further.

In conclusion, goGermline technology eliminates entirely the collateral birth of undesired, non-ES cell-derived offspring.

RESULTS

The *Tsc22d3* Gene

The X-linked gene *Tsc22d3*, also called *Gilz* (D'Adamio *et al*., 1997), encodes a leucine zipper protein that

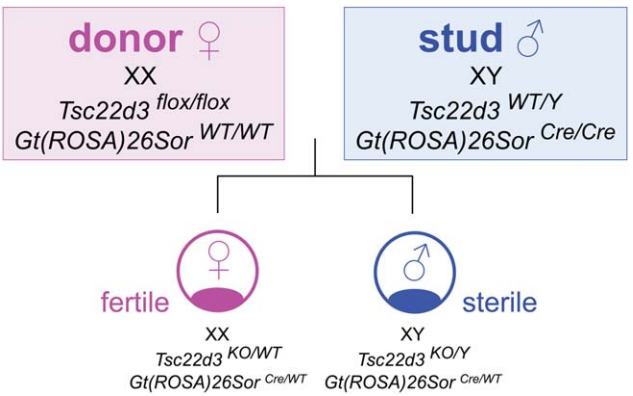

FIG. 1. Schematic of goGermline technology. Blastocysts are produced by mating a superovulated homozygous *Tsc22d3* floxed female (donor) with a homozygous *ROSA26-Cre* male (stud). Male blastocysts develop into sterile males, but fertility can be rescued by injecting ES cells. The resulting chimeras, if fertile, produce exclusively ES cell-derived offspring (100% GLT). Female blastocysts produce fertile females, and these are not bred.

unexpectedly was found to be essential for male fertility. Males hemizygous for a knockout mutation in *Tsc22d3*, from three independently generated strains (Bruscoli *et al*., 2012; Ngo *et al*., 2013a,b; Romero *et al*., 2012; Suarez *et al*., 2012), are sterile due to the inability of spermatocytes to complete the first meiotic division. A few weeks after birth, hemizygous *Tsc22d3* knockout males display Sertoli-cell-only seminiferous tubuli, which are totally devoid of germ cells. Importantly, wild-type germ cells transplanted into the testes of hemizygous *Tsc22d3* knockout males can repopulate seminiferous tubuli (Bruscoli *et al*., 2012), suggesting that these testes can still support normal spermatogenesis. We thus reasoned that the testicular environment of hemizygous *Tsc22d3* knockout males should be conducive to germ cell differentiation of cells that descended from the injected ES cells.

It is obviously not possible to maintain a homogeneous knockout strain if the males of this strain are sterile. Moreover, to streamline the logistics of strain maintenance, we wanted to develop a method whereby no genotyping of the females and males used for blastocyst production is ever necessary. We solved this double challenge by applying a conditional knockout strategy: we cross females homozygous for a floxed *Tsc22d3* mutation with males homozygous for a targeted *ROSA26-Cre* mutation (Fig. 1). Both strains are homozygous viable, healthy, and fertile. Male offspring of this cross carry the *Tsc22d3* mutation on their X chromosome. The *loxP*-flanked segment gets excised in all cells including the germline by the enzymatic action of the Cre recombinase, which is expressed ubiquitously from the *ROSA26* locus. These males are sterile, and can also replace vasectomized males to condition pseudopregnant recipient females.

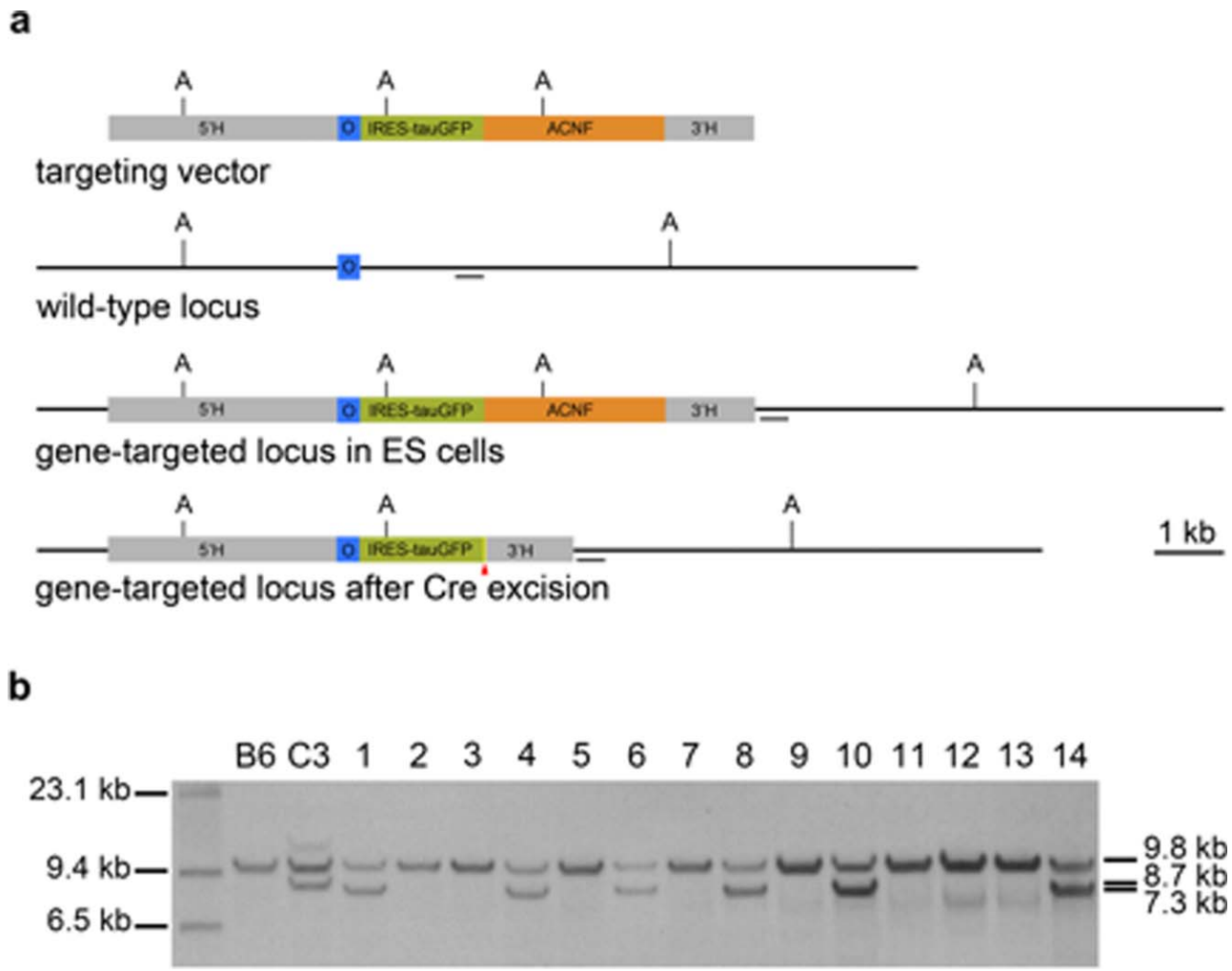

FIG. 2. The OMP-IRES-tauGFP strain. (**a**) Construction of a gene-targeted strain with an OMP-IRES-tauGFP mutation. 5′H, 5′ homology arm; O, the intronless OMP coding sequence; ACNF, neomycin selectable marker; 3′H, 3′ homology arm; A, *ApaL*I restriction site. The *short horizontal bar* denotes the external probe for Southern blot hybridization. The *red triangle* represents the *loxP* site that remains in the locus after self-excision of the *ACNF* cassette during passage through the male germline. The final result is a bicistronic mutation whereby cells that express OMP also express tauGFP. (**b**) Non-radioactive Southern blot hybridization of genomic DNA from the first litter (samples 1–14) produced by a goGermline chimera of clone C3. Six out of 14 mice are heterozygous. Genomic DNA prepared from the liver was digested with *ApaL*I. The fragment representing the wild-type allele is 9.8 kb, the fragment representing the mutant allele in ES clone C3 is 8.7 kb, and the fragment representing the mutant allele in the heterozygous mouse after self-excision of the *ACNF* cassette during passage through the male germline, is 7.3 kb.

goGermline in Setting #1

We tested goGermline technology for two commonly used parental ES cell lines, E14 (Handyside *et al*., 1989) and JM8A3.N1 (Pettitt *et al*., 2009), in the setting of an academic laboratory (Max Planck Research Unit for Neurogenetics). The blastocysts were generated by mating superovulated homozygous *Tsc22d3* floxed, BALB/c × albino-agouti C57BL/6 F1 or F2 females with homozygous *ROSA26-Cre* BALB/c males. (The various strain configurations are described below and in the "Methods" section.) We illustrate the application of goGermline technology in this setting with two projects by way of examples.

In a first project, we injected home-made ES cell clones carrying a targeted bicistronic mutation in the *Omp* gene, which is expressed selectively in mature olfactory sensory neurons (Potter *et al*., 2001). The internal ribosome entry site enables cotranslation of OMP with the axonal marker tauGFP (Fig. 2a). Cells of targeted clones C3, C8, and C47 from parental ES cell line E14 (background 129P2/OlaHsd, chinchilla coat color, $Tyr^{c\text{-}ch}/Tyr^{c\text{-}ch}$ and A^{w}/A^{w}) were injected into, respectively, 22, 34, and 22 blastocysts. We obtained, respectively, 12, 18, and 10 pups (total 40 pups out of 78 transferred blastocysts, or 51%), of which, respectively, 11, 12, and 3 were chimeric (total 26 chimeras out of 40 pups, or 65%). Chimerism could be assessed easily from the coat color differences

Table 1
Exclusive Generation of ES Cell-Derived Offspring with goGermline Technology for Gene-Targeted Clones from Parental ES Cell Line Bruce4

Donor	ES clones	GLT	Transferred blastocysts	Pups born alive	Chimeras born alive	Chimeras at weaning	Male chimeras	Chimeras mated ≥6 weeks	Fertile chimeras	Offspring
BALB/c × albino-agouti B6	106	74 (70)	3,708	1,603 (43)	794 (50)	737 (93)	673 (91)	479 (71)	188 (39)	1,807
C57BL/6	95	60 (63)	2,808	1,013 (36)	636 (63)	545 (86)	543 (99)	403 (74)	121 (30)	1,516
BALB/c × C57BL/6	15	10 (67)	444	172 (39)	100 (58)	87 (87)	87 (100)	61 (70)	24 (39)	195
	216	144 (67)	6,960	2,788 (40)	1,530 (55)	1,369 (89)	1,303 (95)	943 (72)	333 (35)	3,518

The percentage in a given column relates to the number in the previous column. Three configurations for blastocyst donors were tested; the efficiency at each step is comparable. Studs were BALB/c males in all three configurations. Female pups were culled soon after birth, explaining why 95% of weaned chimeras are male. Breeding results are listed only for chimeras that were given the opportunity to breed for ≥6 weeks. As soon as GLT was obtained for a particular ES cell clone, all other chimeras for the same mutation were culled in order to keep the total number of mice generated to a minimum. Therefore, if given a longer time period, more than 35% of male chimeras could have produced offspring. This percentage of 35% thus ought to be regarded as a lower estimate. The 216 gene-targeted ES clones represent 202 different clones; some were used in more than one configuration.

on the albino background. There were, respectively, 4, 7, and 0 male chimeras, and 7, 5, and 3 female chimeras. Three male chimeras from clone C3 were set up for breeding with C57BL/6J females, and two sired 61 offspring (all agouti), of which 26 are heterozygous for the targeted mutation (Fig. 2b). Three male chimeras from clone C8 were set up for breeding with C57BL/6J females, and one sired 13 offspring (all agouti), of which five carried the targeted mutation. The rate of heterozygosity among offspring is consistent with 100% GLT: the goodness of fit (Chi-square test) gives a *P*-value of 0.16 for 31 heterozygotes among 74 offspring.

A second project concerned KOMP clone B07 from parental ES cell line JM8A3.N1 (background C57BL/6N, +/+, and A/a), which carries a knockout mutation in the *Meis2* gene. Using the conventional method of chimera generation by injecting B07 cells into C57BL/6J blastocysts, we had previously obtained only one male chimera with high coat color chimerism. This chimera sired 186 agouti or black offspring when mated with C57BL/6J females. (The JM8A3.N1 parental ES cell line, which is widely used in the KOMP project, has the non-agouti mutation repaired but on only one chromosome, resulting in germline offspring that are agouti or black when male chimeras are crossed with C57BL/6J or C57BL/6N females. All offspring must thus be genotyped.) Only 7 of these 186 offspring were found to be heterozygous, and only 3 of these 7, all females, survived to adulthood. We, thus, surmised that heterozygosity for the *Meis2* mutation causes perinatal lethality. We proceeded to inject B07 cells into 64 goGermline blastocysts. The 19 pups born were all chimeric. Of the 14 male chimeras, 8 were set up for breeding with C57BL/6J females, and 4 sired 77 agouti or black offspring, among which only 3 (one agouti male, one black male, and one black female) were heterozygous; the agouti male died at 2 months. Having confirmed our suspicion of perinatal lethality of *Meis2* heterozygous mice, we sacrificed three C57BL/6J females late in gestation, and genotyped 16 out of 25 embryos as heterozygous. This rate of heterozygosity is consistent with 100% GLT: the goodness of fit (Chi-square test) gives a *P*-value of 0.16 for 16 heterozygotes among 25 embryos. We, thus, capitalized on the exclusive germline transmission of the ES cell-derived genome in goGermline male chimeras: these mice behave effectively as heterozygotes.

goGermline in Setting #2

In order to take full advantage of coat color differences for identifying chimeras and ES cell-derived offspring, and to benefit from high and consistent yields of blastocysts by superovulating hybrid instead of inbred mice, we constructed several specialized strains (see "Methods" section for details.)

We tested three configurations of female blastocyst donors homozygous for the floxed *Tsc22d3* mutation. The first configuration is an F1 or F2 of BALB/c × albino-agouti C57BL/6 (Tyr^c/Tyr^c and A/A at the albino and agouti loci); the second configuration is C57BL/6 (+/+ and a/a); and the third configuration is an F1 of BALB/c × C57BL/6 (Tyr^c/+ and A/a). In all three configurations, studs were homozygous for the *ROSA26-Cre* mutation and BALB/c (Tyr^c/Tyr^c and A/A), and chimeras were bred with C57BL/6 females (+/+ and a/a).

Table 1 summarizes the data obtained at Ozgene with 216 gene-targeted clones from parental ES cell line Bruce4 (background C57BL/6-Thy1.1, +/+, and a/a) (Köntgen *et al*., 1993) representing 115 different alleles. A total of 6,960 transferred blastocysts produced 943 male chimeras that were set up for breeding for at least 6 weeks and sired 3,518 black pups. We obtained GLT for 144 of 216 clones (66.7%). (The real GLT would be higher if chimeras were bred longer, but as soon as heterozygotes are genotyped for a gene-targeted ES clone, all chimeras for the same mutation are culled.) In

FIG. 3. Exclusive transmission of the ES cell-derived genome through a goGermline chimera with low-grade coat-color chimerism. (**Top**) This male chimera was produced by injecting a gene-targeted ES clone from the Bruce4 parental ES cell line (black) into a goGermline blastocyst (albino). The level of coat-color chimerism is minor, and would be deemed insufficient for setting up this chimera for breeding if generated with the conventional method. (**Bottom**) This male chimera sired two litters (here pictured together) when bred with C57BL/6 females (not pictured). All offspring are black and are thus ES cell-derived, demonstrating 100% GLT.

all three configurations, ES cell-derived offspring are black (+/+ and a/a). In the first configuration, which is our preferred configuration and currently our standard practice, host-derived offspring would be agouti (Tyr^{c}/+ and A/a) but have not been seen among 1,807 offspring (which were instead all black), corroborating the extreme stringency of the cell-autonomous defect in spermatogenesis of the *Tsc22d3* mutant phenotype. In the second and third configurations, host-derived offspring would be agouti or black. We identified approximately 50% of heterozygotes among black offspring in the second and third configurations, which is consistent with—but not conclusive of—the absence of leakiness of the *Tsc22d3* mutant phenotype.

We have found repeatedly that low-grade coat-color chimeras, which would not be deemed worthwile setting up for breeding if generated with a conventional method, give 100% GLT (Fig. 3). We surmise that, as there is no competition from host-derived germ cells, the germline of male goGermline chimeras can be colonized efficiently by germ cells that descended from the injected ES cells, regardless of whether these ES cells resulted in high coat-color chimerism.

DISCUSSION

We have here demonstrated that the new goGermline technology can be applied with high efficiency to gene-targeted clones from the commonly used parental ES cell lines E14, JM8A3.N1, and Bruce4. Fertile male chimeras give exclusive, 100% GLT and behave genetically as heterozygotes. The pseudo-heterozygous nature of fertile male chimeras is immensely useful for generating mice with a heterozygous phenotype of diminished viability, such as is the case for the *Meis2* knockout. The method does not afford exclusive transmission of the mutation through the male germline. No genotyping of the two constituent strains of the goGermline technology is required. A major advantage over the Perfect Host approach, as it was described (Taft *et al*., 2013), is that the goGermline technology exploits fully the practical advantages of coat color differences for identifying chimeras and ES cell-derived offspring. Our preferred configuration for blastocyst production is that of homozygous *Tsc22d3* floxed, BALB/c × albino-agouti C57BL/6 F1 females mated with homozygous *ROSA26-Cre* BALB/c males. The yield of blastocysts in this hybrid configuration is high and consistent, and the blastocyts are easy to inject. The albino coat color of these hosts lends itself well to assess coat color chimerism visually for ES cell lines derived from genetic backgrounds with a black (Bruce4, JM8), chinchilla (E14), or agouti (JM8A3, W9.5) coat color. Our extensive validation has not revealed any evidence of leakiness, that is, of hemizygous *Tsc22d3* knockout germ cells producing fertile spermatozoa; the sterility is due to a cell-autonomous defect in spermatogenesis that is extremely stringent. In independent observations, males carrying one of three distinct *Tsc22d3* knockout alleles were reported to be sterile (Bruscoli *et al*., 2012; Ngo et al., 2013b; Romero *et al*., 2012; Suarez *et al*., 2012), consistent with a complete loss of the germline due to an intrinsic failure. Wild-type germ cells transplanted into the testes of hemizygous *Tsc22d3* knockout males can repopulate seminiferous tubuli (Bruscoli *et al*., 2012), suggesting that these testes can still support normal spermatogenesis. Thus, the testicular environment of hemizygous *Tsc22d3* knockout males and of chimeric males developing from injected goGermline blastocysts is conducive to germ cell differentiation, either from transplanted germ cells (Bruscoli *et al*., 2012) or from germ cells that descended from the injected ES cells

(this article). Although there is no reason to doubt the fidelity of sterility, it is cautious to rely on coat color differences when generating chimeras and assessing offspring, if only as quality control for mouse colony management and strain purity.

The complete elimination of the collateral birth of undesired, non-ES cell-derived offspring from breeding conventional chimeras fulfills adequately the reduction imperative of the 3R principle of humane experimental technique with animals (Russell and Burch, 1959). In our current standard operating protocol, a project is considered successful as soon as females mated with chimeras are observed to be pregnant; no further injections need to be scheduled, and these or additional chimeras need not be bred further. In preliminary observations, we have found that it is possible to enrich substantially for chimeras that are fertile (and thus give 100% GLT) by orchidometry, thereby avoiding the housing and attempted breeding of chimeras that turn out to be sterile. Because fertile chimeras behave genetically as heterozygotes, they can be bred directly to Cre or Flp-expressing or other strains, thus eliminating the need for initial colony expansion, hence saving time and further reducing the number of mice used.

CONCLUSION

Recessive mutations in autosomal male fertility genes could be harnessed to develop similar approaches with 100% GLT, but the breeding scheme would be more complex. The location of *Tsc22d3* on the X chromosome is very convenient.

METHODS

Mouse Strains for goGermline Technology

The floxed *Tsc22d3* mutation contains two *loxP* sites that are 1.9 kb apart and flank the last exon of the gene. Gene targeting was carried out in the parental ES cell line Bruce4 (C57BL/6-Thy1.1 background) (Köntgen *et al*., 1993) with G418 selection; a gene-targeted ES clone was injected into F2 of BALB/c x albino-agouti C57BL/6 (Tyr^c/Tyr^c and A/A) blastocysts; and GLT was obtained by crossing chimeras with C57BL/6 mice carrying a *ROSA26-Flp* mutation in order to excise the *FRT*-flanked neomycin selectable marker gene. The floxed *Tsc22d3* mutation devoid of the neomycin selectable marker gene was further maintained in a C57BL/6 background; this is the C57BL/6 strain used in the second and third configurations of Table 1. The floxed *Tsc22d3* mutation devoid of the neomycin selectable marker gene was backcrossed four times to BALB/c and then intercrossed to generate a strain that is homozygous for the floxed *Tsc22d3* mutation and that is also homozygous for a knockout *Tyr* allele at the albino locus and the A allele at the agouti locus; this is the BALB/c strain used in the first and third configurations of Table 1. Separately, the floxed *Tsc22d3* mutation devoid of the neomycin selectable marker gene was backcrossed twice to albino-agouti C57BL/6 mice and then intercrossed to generate a strain that is homozygous for the floxed *Tsc22d3* mutation and that is also homozygous for the $Tyr^{c\text{-}2J}$ allele at the albino locus and the *A* allele at the agouti locus; this is the albino-agouti C57BL/6 strain used in the first configuration of Table 1. In albino-agouti C57BL/6 mice, the wild-type *Tyr* allele at the albino locus and the nonagouti *a* allele at the agouti locus of C57BL/6 have been replaced with, respectively, the albino allele of B6(Cg)-$Tyr^{c\text{-}2J}$/J (The Jackson Laboratory, stock number 000058), and the agouti *A* allele of BALB/c. The *a* and *A* alleles at the agouti locus were distinguished by Southern blotting from ear biopsy genomic DNA. The Tyr^c and $Tyr^{c\text{-}2J}$ alleles were not distinguished by genotyping because both are knockout alleles of the tyrosinase gene and are functionally equivalent; they are here referred to as Tyr^c. The *ROSA26-Cre* mutation was introduced in a BALB/c ES cell line (Noben-Trauth *et al*., 1996), and established and maintained homozygously in a BALB/c background. Strains are available under license from Ozgene.

Generation and Breeding of goGermline Chimeras

Homozygous floxed *Tsc22d3* females were superovulated at age 19–25 days with PMSG and hGC administered 47–48 hr apart. At Ozgene, blastocyts were collected at 3.5 days *post coitum*, injected with ES cells, and transferred to pseudopregnant recipients that are an F1 of CBA × C57BL/6. At the Max Planck Research Unit for Neurogenetics, morulae were collected at 2.5 days *post coitum*, and cultured overnight in KSOM medium; blastocysts were injected with ES cells, and transferred to pseudopregnant recipients of strain CD-1. At Ozgene, most female pups were sacrificed at age 8–10 days, and chimerism of the male pups assessed at 21 days. Males with high coat color chimerism were set up for breeding at age 42 days or later. At Ozgene, these male chimeras were allowed to mate with C57BL/6 females for at least 6 weeks, but when one or more male chimeras gave GLT, the other male chimeras for the same mutation were culled. At Ozgene, all black offspring were genotyped by radioactive Southern blot hybridization. At the Max Planck Research Unit for Neurogenetics, all offspring and embryos were genotyped, by non-radioactive Southern blot hybridization or the polymerase chain reaction.

Gene Targeting at the *Omp* Locus and at the *Meis2* Locus

To create the bicistronic OMP-IRES-tauGFP targeted mutation, the *Omp* coding sequence was generated by

DNA synthesis (GeneArt), and an *Asc*I site was inserted three nucleotides after the stop codon. This synthetic DNA fragment was inserted into generic *Omp* targeting vector pPM9 (Mombaerts *et al*., 1996), which lacks the *Omp* coding sequence. The *IRES-tauGFP-ACNF* cassette was inserted into the *Asc*I site. Gene targeting was carried out in the parental ES cell line E14 (129P2/OlaHsd background), and 90 out of 144 G418-resistant clones (63%) were found to have undergone homologous recombination by genomic Southern blot hybridization. The *ACNF* cassette (Bozza *et al*., 2002; Bunting *et al*., 1999) contains the neomycin selectable marker gene that is self-excised by the Cre/loxP system in the male germline. The MGI allele name is $OMP^{tm17Mom}$. ES cell clone EPD0413_2_B07 was generated by the trans-NIH Knockout Mouse Project (KOMP) (Bradley *et al*., 2012) as IKMC project 26474 with targeting vector DPGS00176_A_D03, and obtained from the KOMP Repository at UC Davis. This ES cell clone was derived from the parental ES cell line JM8A3.N1, and is of the type reporter-tagged deletion allele (with selection cassette) without conditional potential. The MGI allele name is $Meis2^{tm1(KOMP)Wtsi}$. Strains will be available to the research community.

For non-radioactive Southern blot analysis, liver genomic DNA and clone DNA were extracted with Promega Wizard genomic DNA purification kit, and digested overnight with *Apa*LI. The 500 basepair probe was synthesized with the PCR DIG Probe Synthesis kit (Roche) using the primers 5′*GGAGTCCTGCTATCCTGGA*3′ and 5′*GCTCTGGCCACAGCAACTCA*3′. Probe hybridization was overnight in DIG Easy Hyb solution (Roche). The CDP-Star alkaline phosphatase substrate (Roche) was used for probe detection according to the manufacturer's guidelines.

Ethics Statement

All animal studies were carried out in compliance with ethical regulations in Australia and Germany. At Ozgene, mouse experiments were carried out in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Approval came from the Ozgene Animal Ethics Committee. At the Max Planck Research Unit for Neurogenetics, mouse experiments were performed in accordance with the German Animal Welfare Act, the European Communities Council Directive 2010/63/EU, and the institutional ethical and animal welfare guidelines of the Max Planck Research Unit for Neurogenetics. Approval came from the *Regierungspräsidium* Darmstadt and the *Veterinäramt* of the City of Frankfurt.

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AUTHOR CONTRIBUTIONS

F.K. designed the research and analyzed the data. J.L., M. Katidou, I. Chang, and M. Khan performed experiments. J.W. coordinated research and analyzed the data. P.M. supervised the research in his laboratory, managed the collaboration with Ozgene, and wrote the manuscript. All of the authors contributed to editing the manuscript.

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Erklärung zu den Autorenanteilen an der Publikation:

Exclusive transmission of the embryonic stem cell-derived genome through the mouse germline

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Was hat der Promovierende bzw. was haben die Co-Autoren/Autorinnen beigetragen?

(1) zu Entwicklung und Planung
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(2) zur Durchführung der einzelnen Untersuchungen und Experimente
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(3) zur Erstellung der Datensammlung und Abbildungen
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(4) zur Analyse und Interpretation der Daten
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(5) zur Verfassung des Manuskripts
FK: 35%, Promovierender: 5%, MaK: 5%, IC: 5%, MK: 5%, JW: 5%, PM: 40%.

Part V Appendix

List of publications

1. Katidou, M., Grosmaitre, X**., Lin, J.,** Mombaerts, P. (2018). G-protein coupled receptors Mc4r and Drd1a can serve as surrogate odorant receptors in mouse olfactory sensory neurons. **Molecular and Cellular Neuroscience** 88, 138–147.

2. **Lin, J.,** Khan, M., Zapiec, B., Mombaerts, P. (2017). PDGFRA is not essential for the derivation and maintenance of mouse extraembryonic endoderm stem cell lines. **Stem Cell Reports** 9, 1062–1070.

3. **Lin, J.,** Khan, M., Zapiec, B., Mombaerts, P**.** (2016). Efficient derivation of extra-embryonic endoderm stem cell lines from mouse postimplantation embryos. **Scientific Reports** 6, 39457; doi: 10.1038/srep39457.

4. Koentgen, F., **Lin, J.,** Katidou, M., Chang, I., Khan, M., Watts, J., Mombaerts, P. (2016). Exclusive transmission of embryonic stem cell-derived genome through the mouse germline. **Genesis** 54, 326–333.

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Publications

1. Katidou, M., Grosmaitre, X**., Lin, J.,** Mombaerts, P. (2018). G-protein coupled receptors Mc4r and Drd1a can serve as surrogate odorant receptors in mouse olfactory sensory neurons. **Molecular and Cellular Neuroscience** 88, 138–147.

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