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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

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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),

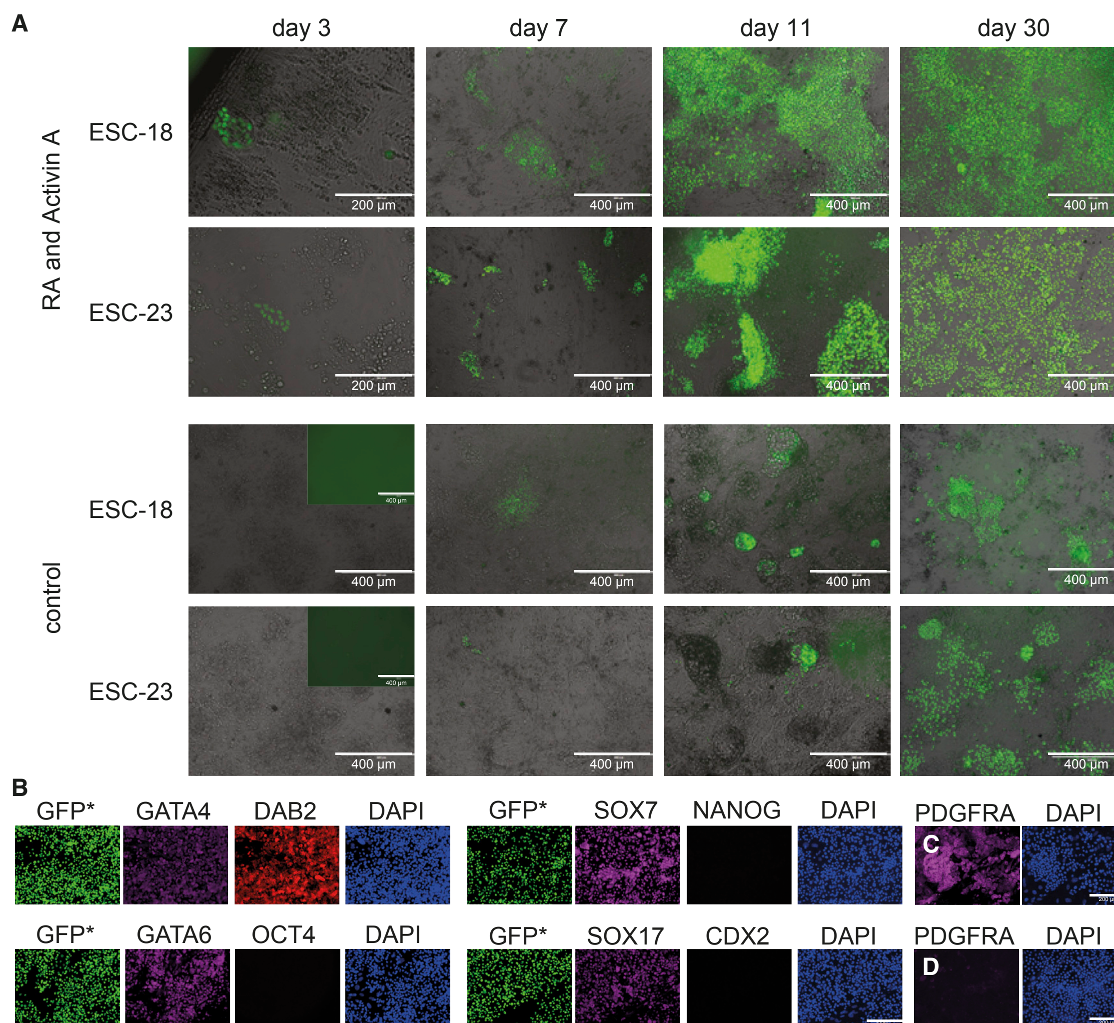

Figure 3. cXEN Cell Lines Converted Chemically from ESC Lines

(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

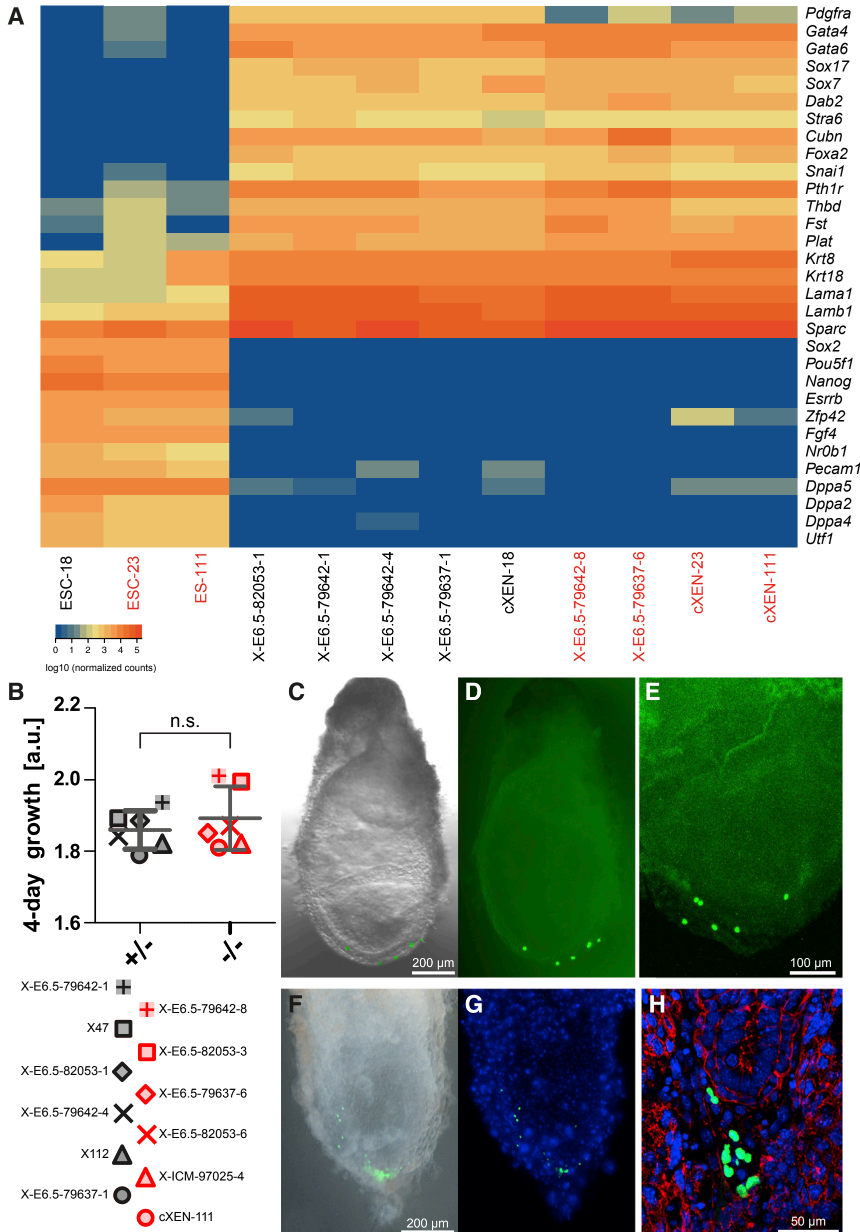

Figure 4. Cell Growth, NanoString Gene Expression Analysis, and Chimeric Embryos

(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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