Identification and characterization of subpopulations in undifferentiated ES cell culture

Yayoi Toyooka1,2,*, Daisuke Shimosato1,3, Kazuhiro Murakami1, Kadue Takahashi1 and Hitoshi Niwa1,3,*

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass (ICM) and the epiblast, and have been suggested to be a homogeneous population with characteristics intermediate between them. These cells express Oct3/4 and Rex1 genes, which have been used as markers to indicate the undifferentiated state of ES cells. Whereas Oct3/4 is expressed in totipotent and pluripotent cells in the mouse life cycle, Rex1 expression is restricted to the ICM, and is downregulated in pluripotent cell populations in the later stages, i.e. the epiblast and primitive ectoderm (PrE). To address whether ES cells comprise a homogeneous population equivalent to a certain developmental stage of pluripotent cells or a heterogeneous population composed of cells corresponding to various stages of differentiation, we established knock-in ES cell lines in which genes for fluorescent proteins were inserted into the Rex1 and Oct3/4 gene loci to visualize the expression of these genes. We found that undifferentiated ES cells included at least two different populations, Rex1+/Oct3/4+ cells and Rex1+/Oct3/4- cells. The Rex1+/Oct3/4- and Rex1+/Oct3/4+ populations could convert into each other in the presence of LIF. In accordance with our assumption that Rex1+/Oct3/4+ and Rex1+/Oct3/4- cells have characteristics similar to those of ICM and early-PrE cells, Rex1+/Oct3/4- cells predominantly differentiated into primitive ectoderm and contributed to chimera formation, whereas Rex1+/Oct3/4- cells differentiated into cells of the somatic lineage more efficiently than non-fractionated ES cells in vitro and showed poor ability to contribute to chimera formation. These results confirmed that undifferentiated ES cell culture contains subpopulations corresponding to ICM, epiblast and PrE.

KEY WORDS: ES cell, Reversibility, Subpopulation, Rex1 (Zfp42), Oct3/4 (Pou5f1), Mouse

INTRODUCTION

Mouse embryos under peri-implantation development have extra-embryonic lineages, and pluripotent cell populations necessary for generating the embryonic part. Fertilized eggs cleave and generate blastomeres, which have equivalent developmental potential. The first cell differentiation occurs at embryonic day (E) 2.5-3.5, and pluripotent cells differentiate into the inner cell mass (ICM), from which embryonic stem (ES) cells are derived, and trophectoderm, which is required for implantation and placentation development (Johnson et al., 2004). Around E4.0, the epiblast and primitive endoderm are derived from ICM cells. After implantation, cavitation occurs by apoptotic cell death of the inner part of the epiblast cells (Coucouvanis and Martin, 1995), and the surviving cells in the outer layer of the epiblast form a columnar epithelium, which we define as primitive ectoderm (PrE), and this gives rise to the embryo proper. ES cells are established from the ICM and epiblast, and may have gained characteristics intermediate between them. ES cells show almost the same proliferation rate (8-12 hours) as epiblast cells (11.5 hours) (Snow, 1977), and strongly express many genes expressed in pluripotent cells in the early embryo, such as Oct3/4, Rex1 and Gbx2 (Sajioh et al., 1996; Rogers et al., 1991; Bulfone et al., 1993). Oct3/4 (also known as Pou5f1) (Yeom et al., 1991), a transcription factor shown to be essential for maintenance of pluripotency (Nichols et al., 1998; Niwa et al., 2000), is expressed continuously in the ICM, epiblast and PrE. Rex1 (also known as Zfp42) is commonly used as a landmark of pluripotency and is strongly expressed in the ICM, but downregulated in the epiblast and PrE (Rogers et al., 1991; Pelton et al., 2002). Hence, we can distinguish the epiblast and PrE from the ICM by Rex1 expression. However, there have been no previous investigations to determine whether these genes are differentially expressed in each ES cell or whether each ES cell has a different pluripotent character.

It is well known that ES cells can mimic cell differentiation events that occur during early mouse development. In vitro, mouse ES cells have been suggested to have the ability to differentiate into primitive endoderm, trophectoderm and many mature somatic cell lineages originating from the embryonic endoderm, mesoderm and ectoderm (reviewed by Keller, 2005; Niwa et al., 2000). Formation of embryoid bodies (EB) using ES cells can mimic peri-implantation development in vitro, and has been mainly exploited for both induction of somatic cells and analysis of the interaction of primitive endoderm and PrE (Keller, 2005). Several studies also demonstrated transient expression of Fgf5 (Haub and Goldfarb, 1991; Hébert et al., 1991), a gene generally used as a PrE marker, in ES cells cultured in adherent culture (Niwa et al., 2000; Shimozaki et al., 2003). These observations suggest that ES cells differentiate into PrE before they convert to mature somatic cells even in culture without EB. We noticed that we could always detect weak Fgf5 expression in undifferentiated ES cell culture, even under selection for Oct3/4 expression (Niwa et al., 2000). As Rex1, Gbx2 (Chapman et al., 1997) and Fgf5 genes differentially expressed in pluripotent tissues in different stages were detected in undifferentiated ES cell culture, we hypothesized that undifferentiated ES cells constitute a heterogeneous population containing pluripotent cells in various stages corresponding from the ICM to PrE. To address this issue, we established knock-in ES cell lines in which the expression of Oct3/4 and Rex1 could be visualized using fluorescent proteins. We found that undifferentiated ES cell culture contained ICM-like...
(Rex1+/Oct3/4+) and early PrE-like (Rex1–/Oct3/4+) cells. These populations showed different differentiation potency in vitro and in vivo, and could convert into each other’s status when cultured in the presence of leukemia inhibitory factor (LIF). These observations suggest that the status of undifferentiated ES cells is in fluctuation between the ICM, epiblast and early PrE.

MATERIALS AND METHODS

Cell culture, construction of knock-in vectors and electroporation

All ES cells were cultured in the absence of feeder cells in Glasgow minimal essential medium (GMEM, Sigma) supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 10−4 M 2-mercaptoethanol, 1× nonessential amino acids and 1000 U/LIF per gelatin-coated dishes.

A knock-in vector for Rex1/4 was designed to replace the coding region of the mouse Oct3/4 gene with an Oct3/4-ECFP fusion gene and IRES (internal ribosome entry site)-puromycin resistance gene, to express Oct3/4-EGFP (or YFP) fusion protein from the recombinant allele. A 4.4 kb fragment containing the coding region of exon 1 to exon 5, and a 2.1 kb fragment containing the untranslated region of exon 5 were amplified from the E14tg2a ES cell genomic DNA and used as the 5′ fragment containing the coding region of exon 1 to exon 5, and a 2.1 kb fragment of exon 4 were amplified from the ES cell genomic DNA and used as the 3′ fragment containing the untranslated region of exon 5 were amplified from the E14tg2a ES cell genomic DNA and used as the 5′ and 3′ homologous regions of the targeting vector, respectively. An Oct3/4-ECFP-IRESpuromycin resistance gene unit was ligated between the two DNA fragments. The resulting vector was linearized by NotI digestion and introduced into the Oct3/4 knock-in ES cell line. Genomic DNAs from blasticidin-resistant colonies were screened for homologous recombination by Southern blotting analyses. For detection of recombination, genomic DNA was digested with EcoRI, and hybridization with a 300 bp probe between the homologous regions of the targeting vector, respectively.

RT-PCR and real-time (quantitative) PCR

Total RNA was extracted using Trizol (Invitrogen) in accordance with the manufacturer’s protocol. Total RNA (1.0 μg) was then subjected to oligo-dT-primed reverse transcription (RT) with ReverTra Ace Kit (Toyobo, Osaka, Japan). RT-PCR was performed using DNA polymerase (GeneTaq, NipponGene) on an iCycler (BioRad). For real-time PCR analysis, total RNA was prepared using Trizol and CDNs were synthesized with oligoDT primer by ReverTraAce first strand synthesis kit (Toyobo). Q-PCR reactions were performed using the ExTaq SYBR Green Supermix (Takara) and an iCycler System (Bio-Rad). The amount of target RNA was determined from the appropriate standard curve and normalized relative to the amount of Gapdh mRNA. Gene-specific primers for RT-PCR and Q-PCR were designed based on published sequences (Table 1).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 30 minutes at 4°C, washed with PBS containing 0.2% Triton X-100 for 15 minutes at room temperature, incubated with PBS containing 2% FCS for 20 minutes to block non-specific reaction, and incubated with anti-Nanog rabbit polyclonal antibody (RCAB0001P, ReproCell), anti-Klf4 rabbit polyclonal antibody, (sc-20691, Santa Cruz) anti-Tbx3 rabbit polyclonal antibody (originally raised in our laboratory with GST-Tbx3 fusion protein), anti-Esrrb monoclonal antibody (Perseus, pp-H6705-00) or anti-Foxd3 monoclonal antibody (MAB2819, R&D Systems) for overnight at 4°C. Dilution ratio of first antibodies are:

<table>
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<tr>
<th>Gene</th>
<th>Sense primer</th>
<th>Antisense primer</th>
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<tr>
<td>Oct3/4</td>
<td>CAGAAGGAGCTGAGAACGATTGTGCG</td>
<td>AGATGGTGGCTGTGGCTGAAC</td>
</tr>
<tr>
<td>Rex1</td>
<td>CGAGTGGGCTGCTGGCTGAAC</td>
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<td>Gapdh</td>
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Table 1. Primer sequences (5′-3′) for RT-PCR and Q-PCR

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Subpopulations in undifferentiated ES cell culture

RESULTS

Rex1\(^+\) and Rex1\(^-\) subpopulations in undifferentiated ES cell culture

To determine whether subpopulations corresponding to ICM and PrE exist in ES cell culture, we established a system for detecting ICM and PrE from ES cells using Oct3/4 and Rex1 genes as molecular markers. We established knock-in ES cell lines in which the expression of Oct3/4 and Rex1 could be visualized by CFP and GFP fluorescence, respectively (Fig. 1). We observed double-knock-in ES cells under puromycin selection (selection for Oct3/4\(^+\) and Rex1\(^+\)) on gelatin-coated dishes, and found that Rex1\(^+\) positive and -negative populations existed in Oct3/4\(^+\) and Rex1\(^+\) morphologically undifferentiated ES cell populations (Fig. 2A,B). Most colonies mainly composed of Rex1\(^+\) showed flat morphology and had both CFP and GFP fluorescence (Fig. 2C-E). Colonies in which CFP-positive and GFP-negative were dominant showed compact morphology and had both CFP and GFP fluorescence (Fig. 2C-E). The average proportion of Rex1\(^+\) (GFP) positive and negative cells was about 9:1 in culture medium with serum (GMEM supplemented with 10% FCS). These observations indicate that Oct3/4\(^+\)/positive ES cells are heterogeneous and contain at least two subpopulations. We examined the expression of platelet endothelial cell adhesion

Fig. 1. Structure of knock-in vectors for Oct3/4 and Rex1

Structure of knock-in vector for Oct3/4 constructed to replace the coding region of the mouse Oct3/4 gene with Oct3/4-ECFP (or YFP) fusion gene and IRES-puromycin resistance gene to express Oct3/4-ECFP (or YFP) fusion protein from the recombinant allele, and structure of knock-in vector for Rex1 for insertion of an ECFP (or herpes simplex virus 2 thymidine kinase gene) and IRES-blasticidin resistance gene into exon 4 of the mouse Rex1 gene. Black boxes represent coding regions. Southern blotting analyses of the Oct3/4 locus and the Rex1 locus are shown at the bottom. Genomic DNA was digested with EcoRI for analyses of the Oct3/4 locus, a 300 bp probe from intron 5 produced an 11.6 kb band from the wild-type locus and a 10.3 kb band from the targeted locus. For analyses of the Rex1 locus, genomic DNA was digested with EcoRI, and hybridization with a 300 bp probe produced a 7.9 kb band from the wild-type locus and a 5.7 kb (5.9 kb for tk2 vector) band from the targeted locus. BSD, blasticidin S deaminase; E, EcoRI; P, positions of probes; Puro, puromycin-N-acetyltransferase; S, Spel; tk2, herpes simplex virus 2 thymidine kinase (HSVtk2).
molecule 1 [PECAM-1 (Pecam1)] in Rex1+ and Rex1− populations. PECAM-1 protein was detected specifically in the ICM of the 3.5 dpc blastocyst and was not detected in PrE, and was suggested to be another useful marker of ICM cells (Robson et al., 2001). Using anti-PECAM-1 antibody, we found that expression of PECAM-1 was correlated positively with expression of Rex1 (Fig. 2K); this represents evidence that Rex1+ ES cells have ICM-like properties.

**Rex1+ and Rex1− subpopulations have different patterns of gene expression**

Detailed gene expression patterns of sorted Rex1+ and Rex− subpopulations were examined by means of Q-PCR. The data show that Tbx3, a gene specifically expressed in the ICM of blastocysts (Chapman et al., 1996), was significantly abundant in the Rex1+ fraction, and genes that were strongly expressed in embryos in preimplantation stages and downregulated in post-implantation embryos (based on EST data of NCBI UniGene), such as Dppa3, Klf4, Esrrb and Tcl1, were also highly expressed in Rex1+ cells (Fig. 3A). By contrast, transcripts of Fgf5, brachyury (T) and Eomes, which were upregulated in the PrE and early germ layers (Pelton et al., 2002; Tesar et al., 2007), were detected at higher levels in the Rex1− population (Fig. 3A). We could also observe these biased expression patterns in Nanog, Klf4, Tbx3 and Esrrb in culture of Rex1-GFP knock-in ES cells by immunostaining with antibodies for them (Fig. 3B). Nanog, Klf4, Tbx3 and Esrrb tended to be detected in EGFP+ (Rex1+) cells, whereas Foxd3 did not show such a bias, coincident with the result of Q-PCR. Expression levels of Oct3/4 and Sox2 associated with pluripotency were not much different between the two populations (Fig. 3A). These expression patterns of early genes support our hypothesis that Rex1+ and Rex1− populations have features of pluripotent cell population in pre- and post-implantation stages, respectively.

**Reversibility of Rex1+ and Rex1− populations**

To examine the characteristics of Rex1+/Oct3/4+ ES cells, we purified both populations by flow cytometry and grew the cells in culture. Oct3/4-CFP/Rex1-GFP double-knock-in ES cells were incubated under puromycin selection, and then Rex1+ or Rex1− cells were purified using GFP fluorescence. We cultured purified Rex1+ and Rex1− cells at more than 98% purity and found that a purified Rex1+ population generated a Rex1− population, and that a Rex1− population emerged from a purified Rex1+ population in a comparatively short period (Fig. 4A). To exclude the possibility that this regeneration of another subpopulation was merely due to contamination, we also performed single-cell culture of Rex1+ and Rex− cells. Rex1+ cells arose from a single Rex1− cell within 2-3 days (Fig. 4B), and finally showed the same proportion as the parental double-knock-in cell line after culture for 10-14 days (data not shown). These observations indicated that Rex1+ and Rex1− cells have the ability to convert into each other’s status.

**The Rex1+ population predominantly contributes to embryonic tissues**

It has already been shown that the ICM and epiblast can contribute to chimera formation, whereas PrE cannot (Gardner, 1971; Rossant, 1977; Bedington, 1983; Brook and Gardner, 1997). We performed blastocyst injection of Rex1+ and Rex1− populations to examine their capacity for contributing to embryonic tissue in vivo. Rex1+/Oct3/4 double-knock-in ES cells were marked by forced expression of the DsRedT4 gene driven by the CAG promoter, and EGFP+ and EGFP− cells were purified from puromycin-resistant cells. While 5 of 22 embryos from blastocysts injected with Rex1+ cells showed poor ability as PrE. We also noted that pseudopregnant mice into which blastocysts injected with Rex1− cells were transferred had more vacant decidua (ratio of embryos/decidua: 41/120) than those with blastocysts injected with Rex1+ cells (22/42). This indicated that embryos injected with Rex1− cells tend to degenerate, in contrast to those injected with Rex1+ cells.
Differentiation ability of Rex1+ and Rex1− subpopulations in vitro

Considering the expression pattern of the Rex1 gene in vivo, the ES cell population expressing Rex1 could be thought to correspond to the ICM in vivo and the Rex1− cell population may correspond to early PrE. For comparison of the differentiation ability of ICM-like and PrE-like populations in vitro, we established a cell line in which one Rex1 locus was replaced by herpes simplex virus 2 thymidine kinase (HSVtk2) fused to an IRES-blasticidin resistance gene cassette using an Oct3/4-YFP knock-in ES cell line. This cell line allowed us to purify Rex1+ and Rex1− cells without cell sorting, because Rex1-expressing (ICM-like) cells can be eliminated by addition of gancyclovir (GANC) from Oct3/4-positive cells selected by puromycin, whereas they can be enriched by addition of blasticidin S (Fig. 1, Fig. 6A). We first checked expression of genes that can be used as markers of PrE in cells selected as PrE-like cells in this system. Rex1-negative cells selected with GANC and puromycin strongly expressed Fgf5 and T, which began to be expressed in the developing embryonic endoderm in vitro. Whereas withdrawal of LIF induced robust expression of Gata4 and Sox7, marker genes for primitive endoderm in the control fraction, a much lower level of expression of these genes was induced in the PrE-like fraction (Fig. 6C). This suggested that PrE-like cells almost lack the ability to differentiate into primitive endodermal cell lineage. Rex1− cells selected with GANC could also adopt the morphology of Rex1+ ES-like colonies when they were re-seeded in medium without GANC followed by addition of blasticidin S. We confirmed that these ‘reverted’ Rex1+ cells could regain not only the Nanog and Tbx3 expression levels, but also differentiation ability for primitive endodermal lineage (Fig. 6C). Similar reversibility of the phenotypes respond to withdrawal of LIF was found in Rex1+ and Rex1− populations isolated by FACS sorting (data not shown).

In vivo, PrE cells are pluripotent cells that do not differentiate into extra-embryonic cell lineages but into somatic and germ cell lineages. Therefore, we supposed that PrE-like cells differentiate into somatic cell lineage more efficiently than ICM-like populations. Next, we examined the abilities of ES cells selected by Oct3/4 expression and enriched PrE-like (Rex1−) cells to differentiate into somatic cell lineages using the culture system for inducing mesodermal cell lineage differentiation (Nishikawa et al., 1998). Cells were incubated for 1-4 days on type-IV-collagen-coated dishes for induction of differentiation, then stained with anti-E-cadherin antibody and Flk1 antibody, and the frequencies of E-cadherin-negative and Flk1-positive cells were measured as markers for primitive endoderm and mesoderm, respectively (Figs. 6B, 7B). We also performed neural induction using the neural induction system developed by Ying et al. (Ying et al., 2003b). Although they used an ES cell line that reported Sox1 expression as GFP fluorescence, we used an antibody to the pan-neural marker NCAM to detect neural cells (Fig. 7C). We obtained similar results to those reported elsewhere for mesoderm induction, with detection of an increasing number of NCAM+ cells in the Rex1−/Oct3/4+ PrE-like fraction, which was composed mainly of Rex1−/Oct3/4+ cells (Fig. 7B). We also examined the subpopulations in undifferentiated ES cell culture with respect to the expression levels of early genes in Rex1+ and Rex1− populations. The averages of values from RNA of Rex1+ and Rex1− cells sorted three times independently are shown in the graph (Fig. 3). Immunostaining of Rex1+/EGFP/Oct3/4-ECFP double-knock-in cells with antibodies for proteins expressing in the peri-implantation embryos. Fluorescence of antibody reaction is shown as red signal (Alexa 594) for Nanog, Klf4 and Tbx3, or purple signal (Alexa 647) for Esrrb and Foxd3.

**Fig. 3. Expression patterns of genes associated with early development in mouse Rex1+/− populations.** (A) Ratio of expression levels of early genes in Rex1+ and Rex1− populations were examined by Q-PCR. The averages of values from RNA of Rex1+ and Rex1− cells sorted three times independently are shown in the graph. (B) Immunostaining of Rex1+/EGFP/Oct3/4-ECFP double-knock-in cells with antibodies for proteins expressing in the peri-implantation embryos. Fluorescence of antibody reaction is shown as red signal (Alexa 594) for Nanog, Klf4 and Tbx3, or purple signal (Alexa 647) for Esrrb and Foxd3.

**Ratio of Rex1+/− populations was biased under different culture conditions**

In addition, we found that the proportion of the two populations was significantly different between ES cells cultured under serum-free conditions reported by Ying et al. (N2B27 medium with Bmp4 and LIF) and under those reported by Ogawa et al. [GMEM
medium with LIF, containing KSR and adenocorticotropic hormone peptide (ACTH) instead of serum] at clonal density. In KSR/ACTH medium, ES cells mainly formed packed colonies (Fig. 8A), and most cells (~96%) were Rex1+ cells (Fig. 8B). Cells cultured in N2B27 mainly formed flat colonies (Fig. 8A) containing cells showing low levels of Rex1 expression (or negative) at a high proportion (~25-30%; Fig. 8B). These observations suggested that these culture conditions supported maintenance or proliferation of different subpopulations in ES cells with differential efficiency.

**DISCUSSION**

Here, we reported that there are subpopulations of Rex1+/Oct3/4+ cells and Rex1–/Oct3/4+ cells in undifferentiated ES cells, and that they converted into each other when cultured in the presence of LIF. According to the gene expression pattern and differentiation capacity in vitro and in vivo, Rex1+/Oct3/4+ cells were assumed to be analogs of ICM and epiblast stages, and Rex1–/Oct3/4+ cells were estimated to be equivalent to early PrE (Fig. 9).

It has been reported that primitive endoderm is necessary for the establishment of PrE (Li et al., 2001; Li et al., 2002; Fassler and Meyer, 1995; Smyth et al., 1999). The basement membrane deposited from primitive endoderm is necessary for survival and polarization of adjacent outer epiblast cells to form the columnar structure of PrE, and inner cells that fail to attach to the basement membrane undergo apoptosis (Coucouvanis and Martin, 1995). In early mouse embryos, extracellular matrices (ECMs), such as laminin 1 and 10, nidogen 1 and 2, perlecan, agrin and collagen IV, have been reported to be present as basement membrane components. Of these, laminin 1 is well known to be a crucial factor for early development (Smyth et al., 1999; Schéele et al., 2005). Embryoid bodies that lack expression of laminin 1 failed to form the columnar structure of PrE (Li et al., 2001; Li et al., 2002). The results of targeted disruption of integrin B1, a receptor of laminin, and integrin-linked kinase (ILK), also demonstrated their importance in PrE formation (Fassler and Meyer, 1995; Sakai et al., 2003). Although we found that a small population of PrE cells emerged in ES cell culture cell-autonomously without any treatment,
cell differentiation is irreversible in real embryos, and epiblasts and PrE cells do not regain the character of ICM in vivo. A similar reversible phenotype observed in vitro was reported by Suzuki et al. (Suzuki et al., 2006). They induced mesoderm progenitor cells from ES cells by reduction of LIF concentration in medium. Even the mesoderm progenitor cells contributed only to mesodermal tissue when injected into blastocysts, and they could repopulate undifferentiated ES cells in vitro (Suzuki et al., 2006). It may be possible that although the nature of pluripotent or stem cell lineages in the early embryo is potentially reversible, signals from the ECM in the basement membrane function as a niche. The ECM secreted from the primitive endoderm may fix the PrE status, and cause the cells to retain the PrE character.

We showed that Rex1+/Oct3/4+ cells and Rex1+/Oct3/4+ cells have different differentiation ability in vivo and in vitro. Rex1+/Oct3/4+ cells could contribute to chimera formation predominantly. It has been shown that the ICM and epiblast can contribute to chimera formation, while PrE cannot (Gardner, 1971; Rossant, 1977; Beddington, 1983; Brook and Gardner, 1997). The results of chimera experiments in the present study were consistent with their in vivo data, suggesting that Rex1+ cells are equivalent to cells in the ICM and epiblast in vivo. We also noted that the survival rate of embryos injected with Rex1+ cells was much lower than that of embryos injected with Rex1+ cells. We assumed that ES cells corresponding to different developmental stages are not only omitted from embryogenesis, but may impede the normal process of development when injected into the blastocyst. Additionally, we demonstrated the poor ability of Rex1+/Oct3/4+ cells to differentiate into primitive endoderm whereas these cells predominantly differentiated into somatic lineages using a negative selection system, and also that their differentiation ability seemed interchangeable because their ability for extra-embryonic lineage was restored in reverted Rex1+ populations. These observations were also consistent with the behavior of PrE cells in the context of development. We first performed in vitro experiments with sorted Rex+ and Rex− cells, but we could not obtain clear results regarding the difference in differentiation ability between Rex+ and Rex− populations. We supposed that this was caused by the quick-reversible phenotype of both populations in vitro, and we attempted to take advantage of a negative selection system. The results of chimeric analysis and in vitro experiments taken together indicated that Rex+/Oct3/4+ cells and Rex−/Oct3/4+ cells showed different differentiation abilities and that they were similar in characteristic to ICM and PrE, respectively, in vivo.

The generation and characterization of PrE-like cells in vitro have been reported. Rathjen and colleagues reported that they generated early primitive ectoderm-like (EPL) cells, which showed the character of PrE-like cells, from ES cells (Rathjen et al., 1999; Lake et al., 2000). They obtained EPL cells by culturing ES cells with MEDII medium, which contained the conditioned supernatant of the hepatocyte cell line HepG2. They also demonstrated the lack of ability to differentiate into primitive endoderm, inability to contribute to chimera formation, and reversible phenotype in response to withdrawal of MEDII. EPL cells expressed Oct3/4 and Fgf5, and Rex1 at very low levels. Although it is difficult to compare our data to those of Rathjen’s group as they did not report data from analysis at the single-cell level nor used a stem cell selection system, we suppose that their EPL cells would be mainly composed of the Rex+ population.

Furusawa et al. also reported variation in the expression of platelet endothelial cell adhesion molecule 1 (PECAM-1) and stage-specific embryonic antigen (SSEA-1; also known as F4u4 – Mouse Genome Informatics) in ES cell culture. Three sorted populations in ES cells, PECAM-1+/SSEA-1+, PECAM-1+/SSEA-1− and PECAM-1−/SSEA-1−, could give rise to two other populations; PECAM-1−/SSEA-1+ cells predominantly contribute epiblast cells in chimeras, and could differentiate into primitive endoderm in vitro much more efficiently than the other two populations (Furusawa et al., 2004). We confirmed that PECAM-1 expression is nearly consistent with Rex1 expression (Fig. 2K), and thus the Rex+ subpopulation was thought to contain SSEA-1+ and SSEA-1− subpopulations. Furusawa and his colleagues showed that the PECAM-1−/SSEA-1− population showed less ability to differentiate into Fgf5+ cells compared with the PECAM-1+/SSEA-1+ population in vitro, which does not match the characteristics of the Rex+ population we observed (Furusawa et al., 2004). This discrepancy might be due to contamination of differentiated cells into the PECAM-1−/SSEA-1− population, as they did not use any selection system to exclude differentiated cell populations.

Recently two independent groups reported that pluripotent stem cells could be established from mouse post-implantation embryos after E5.5 (termed EpiSCs) (Tesar et al., 2007; Brons et al., 2007). EpiSCs were derived from late epiblast or PrE, probed at very low levels. Although it is difficult to compare our data to those of Rathjen’s group as they did not report data from analysis at the single-cell level nor used a stem cell selection system, we suppose that their EPL cells would be mainly composed of the Rex+ population.

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Table 2. Comparison of chimerisms between Rex+ and Rex− ES cells

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<th>Injected cells</th>
<th>No. of transferred embryos</th>
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<td>Rex1(GFP)+</td>
<td>65</td>
<td>42</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>Rex1(GFP)−</td>
<td>157</td>
<td>120</td>
<td>41</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 5. Blastocyst injection was performed with mouse Rex1+ and Rex1− ES cell populations to compare their capacities for contributing embryonic tissue. (A–D) Rex1/Oct3/4 double-knock-in ES cells were marked by forced expression of the DsRedT4 gene driven by the CAG promoter, and EGFP+ and EGFP− cells were purified after culture under puromycin selection. Some of the 10.5 dpc embryos derived from blastocysts injected with the Rex1+ fraction showed DsRed fluorescence. The embryo on the left has cells showing strong fluorescence. Embryos injected with the Rex1− fraction did not show DsRed fluorescence.
they have a very similar character to that of the *Rex1* population we observed (refer to Fig. 3A). The crucial difference between EpiSCs and the *Rex1* population in ES cells is that *Rex1* cells can keep their status only very transiently but change into *Rex1* within a short period, whereas EpiSCs seem to be able to keep their status consistently. EpiSCs had quite a different pattern of histone methylation in the promoter region of several genes from that of ES cells (Tesar et al., 2007); it might be possible that such an epigenetic status of cells is crucial to lock the reversibility and continue self-renewal.

**Fig. 6.** Examination of mouse ES cells for differentiation ability of *Rex1*/*Oct3/4* cells selected with thymidine kinase gene/gancyclovir system into primitive endoderm lineage. (A) Thymidine kinase gene knock-in ES cell lines under selection for puromycin and gancyclovir (GANC). (Left) Cells selected with puromycin (selection for *Oct3/4* cells); (right) cells selected with both puromycin and GANC (selection for *Oct3/4* and exclusion of *Rex1* cells). (B) Expression of marker genes was examined by RT-PCR in puromycin and GANC/puromycin double-selected fractions. (C) Expression of primitive endoderm marker genes in differentiated *Rex1*+, *Rex1* and ‘reverted’ *Rex1* cells by withdrawal of LIF. Before starting induction of differentiation, *Rex1* cells were selected with blasticidin S whereas *Rex1* cells were selected with GANC and puromycin for 1 week. Reverted *Rex1* cells were derived from *Rex1* cells re-seeded and cultured without GANC for 3 days, and then cultured with blasticidin S for 4 days. Scale bar: 100 μm.

**Fig. 7.** Differentiation ability of *Rex1*/*Oct3/4* cells into mesodermal and neural lineages. (A) Dot-plots of analysis at day 0 and day 4 for mesodermal induction from unselected *Rex1*-tk2 knock-in ES cells. Percentage of Flk1+/E-cadherin cells (indicated by rectangles) was measured by analyzer. (B) The results of induction of mesoderm from *Rex1*/*Oct3/4* and *Rex1*/*Oct3/4* populations selected with GANC and puromycin. Note that *Rex1*/*Oct3/4* (PrE-like cells) differentiated into mesodermal cells more efficiently and with faster kinetics than non-fractionated ES cells. (C) Analysis of induction of neural cells (dot-plot on day 4). Anti-NCAM antibody and Alexa-633-conjugated anti-goat IgG antibody were used as first and second antibodies, respectively, for measurement of the proportion of neural cells. Green line, control ES cells stained with only secondary antibody; blue line, fraction of *Oct3/4* cells on day 5; red line, enriched *Rex1*/*Oct3/4* fraction on day 5. (D) Summary of experiment for induction of neural cells on days 2, 4 and 5.
Subpopulations in undifferentiated ES cell culture

We also showed that the proportions of each population were significantly different between ES cells cultured under serum-free conditions reported by two different groups: N2B27 supplied with LIF and Bmp4 (Ying et al., 2003b), and KSR-based medium with ACTH and LIF (Ogawa et al., 2004). In N2B27 medium, ES cells tended to retain the epiblast-PrE state, which was unexpected because serum-free conditions were thought to suppress the appearance of differentiated cells, and as a consequence ES cells could generally maintain the ICM-like state. Ying and colleagues reported that inputs from LIF and Bmp4 contained in their medium were crucial for the maintenance of pluripotency; LIF inhibited cell differentiation toward the mesodermal and endodermal lineage, whereas Bmp4 suppressed differentiation toward neuroectoderm (Ying et al., 2003b). It is possible that ES cells actively maintained the ICM-like state in KSR medium supplemented with ACTH and LIF, whereas they remained in the undifferentiated state passively as a consequence of inhibition of commitment to the differentiation pathway to any cell lineage in N2B27 medium with Bmp4 and LIF. This result implies that these subpopulations require different factors and signal transduction pathways for maintenance of their state, and comparison and verification of each component of these serum-free media may provide some insight into the factor(s) essential for the transition between Rex1+ and Rex1− status. These observations also indicated that different subpopulations could be dominant under different culture conditions, and this may considerably influence the results of the experiment in some situations.

There have been a few reports regarding the regulation of Rex1 gene expression. Ben-Shushan et al. reported that Oct3/4 regulates the Rex1 promoter in F9 EC cells (Ben-Shushan et al., 1998). Shi et al. showed that two transcription factors essential for maintenance of pluripotency, Nanog (Chambers et al., 2003; Mitsui et al., 2003) and Sox2 (Avilion et al., 2003), activate expression of Rex1 directly in F9 cells (Shi et al., 2006). They also showed that overexpression of Nanog triggers Rex1 expression in P19 cells, which originally did not express Rex1, indicating that Nanog plays a crucial role in regulation of expression of Rex1 in ES cells. It is of interest to examine whether overexpression of Nanog and/or Sox2 prevents the fluctuations of Rex1 expression in ES cells. Rex1−/− F9 cells were able to differentiate into only parietal endoderm, whereas wild-type F9 cells could differentiate into primitive, visceral and parietal endoderm. Rex1 was assumed to regulate the differentiation of F9 cells along several distinct cell lineages in the early embryo (Thompson and Gudas, 2002). However, we found that Rex1−/− ES cells could be established and differentiate normally both in vitro and in vivo, although induction of some of the visceral marker genes were affected, and we could produce Rex1−/− mice by a conventional gene-targeting strategy (S. Masui, S. Ohtsuka, R. Yagi, K.T., M. S. H. Ko and H.N., unpublished). Thus, Rex1 may not be essential for embryogenesis and maintenance of pluripotency of ES cells.

Although it has already been reported that differentiation into primitive endoderm can be induced by upregulation of Oct3/4 or forced expression of GATA factors (Fujikura et al., 2002; Shimosato et al., 2007; Niwa et al., 2000), whereas differentiation into trophoderm is induced by downregulation of Oct3/4 and activation of Cdx2 (Niwa et al., 2000; Niwa et al., 2005), intrinsic factors crucial for differentiation into somatic lineages have not yet been identified, and induction of somatic cell lineages can be achieved at present by the withdrawal of LIF from culture medium. Using our system to detect the early PrE-like transient population in ES cell culture, it may be possible to identify the factor(s) crucial for transition of ICM to PrE in vivo.

**Fig. 8.** Morphology and results of flow cytometric analysis of mouse Rex1/Oct3/4 double-knock-in ES cells cultured in different serum-free media under puromycin selection (selection for Oct3/4 expression). (A) Morphology and GFP fluorescence of cells cultured in GMEM supplemented with KSR, ACTH and LIF (above) and those cultured in N2B27 medium supplemented with Bmp4 and LIF (below). (B) Flow cytometric analysis of cells cultured in GMEM+FCS, +LIF (left), in GMEM+KSR, +ACTH, +LIF (center) and in N2B27 medium +Bmp4, +LIF (right). Scale bar: 100 μm.

**Fig. 9.** Illustration showing the expression patterns of mouse marker genes expressed in the pluripotent cell population at each embryonic stage (upper), and the subpopulation in ES cells (lower) that could be detected by expression of these marker genes. ICM-like (Rex1+) and early PrE-like (Rex1−) populations have different abilities for in vitro differentiation and could convert into each other under normal conditions for ES cell maintenance.
We thank Dr Shin-Ichi Nishikawa and Satomi Nishikawa (RIKEN, CDB, JAPAN) for providing us with several materials necessary for the culture system to induce mesodermal lineage. We also thank Ms Etsuko Hasegawa (RIKEN, CDB) for her help with the cell sorting.

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Ben-Shushan, E., Thompson, J. R., Gudas, L. J. and Bergman, Y. (2003). BMP induction of mesodermal lineage. We also thank Ms Etsuko Hasegawa (RIKEN, CDB) for her help with the cell sorting.

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