View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Cell

Interaction between Oct3/4 and Cdx2 Determines Trophectoderm Differentiation

Hitoshi Niwa,[1,2,3,*] Yayoi Toyooka,[1] Daisuke Shimosato,[1,2] Dan Strumpf,[4] Kadue Takahashi,[1] Rika Yagi,[1] and Janet Rossant[4]
[1] Laboratory for Pluripotent Cell Studies, RIKEN Center for Developmental Biology (CDB), 2-2-3 Minatojima-minamimachi, Chu-o-ku, Kobe, Hyogo 650-0047, Japan
[2] Laboratory for Development and Regenerative Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunokicho, Chu-o-ku, Kobe, Hyogo 650-0017, Japan
[3] CREST (Core Research for Evolutional Science and Technology), Japan Science and Technology Agency, Honcho 4-1-8, Kawaguchi, Saitama 332-0012, Japan
[4] Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada
*Contact: niwa@cdb.riken.jp
DOI 10.1016/j.cell.2005.08.040

SUMMARY

Trophectoderm (TE), the first differentiated cell lineage of mammalian embryogenesis, forms the placenta, a structure unique to mammalian development. The differentiation of TE is a hallmark event in early mammalian development, but molecular mechanisms underlying this first differentiation event remain obscure. Embryonic stem (ES) cells can be induced to differentiate into the TE lineage by forced repression of the POU-family transcription factor, *Oct3/4*. We show here that this event can be mimicked by overexpression of *Caudal-related homeobox 2* (*Cdx2*), which is sufficient to generate proper trophoblast stem (TS) cells. *Cdx2* is dispensable for trophectoderm differentiation induced by *Oct3/4* repression but essential for TS cell self-renewal. In preimplantation embryos, *Cdx2* is initially coexpressed with *Oct3/4* and they form a complex for the reciprocal repression of their target genes in ES cells. This suggests that reciprocal inhibition between lineage-specific transcription factors might be involved in the first differentiation event of mammalian development.

INTRODUCTION

Trophectoderm (TE) is the first differentiated cell lineage to arise in mammalian embryogenesis. Mouse zygotes cleave three times to generate 8-cell stage embryos. After the third cleavage, the blastomeres undergo a morphological change known as compaction. The next round of cell divisions tends to occur along the apical-basal axis of the blastomeres, resulting in the formation of a 16-cell morula consisting of small inner cells enclosed within larger outer cells. Most of the outer cells are then epithelialized and become TE, whereas the inner cells go on to generate the inner cell mass (ICM) in blastocysts (Fleming, 1987). Cell-fate analyses revealed that the ICM gives rise to all of the embryonic cells and the extraembryonic endoderm, whereas TE forms the embryonic portion of the placenta, a structure unique to mammalian development (Pedersen et al., 1986; Fleming, 1987). Therefore, the differentiation of TE can be regarded as a hallmark event in mammalian early development.

We previously reported that the pluripotent embryonic stem (ES) cells derived from the ICM can be induced to undergo differentiation toward the TE lineage by forced repression of a POU-family transcription factor, *Oct3/4*, whereas its overexpression induces differentiation mainly to extraembryonic endoderm (Niwa et al., 2000). It had previously been suggested that mouse ES cells possess limited ability to form TE and extraembryonic endoderm because they contribute to these lineages at low frequencies when they are injected into blastocysts to generate chimeric embryos (Beddington and Robertson, 1989); but our study conclusively demonstrated that ES cells can be caused to differentiate into these extraembryonic lineages by controlling the function of genes involved in these differentiation events.

It was recently shown that *Caudal-related homeobox 2* (*Cdx2*) is involved in TE formation at the blastocyst stage in mice. The expression of *Cdx2* in the pre- and early postimplantation embryos is tightly restricted in the TE lineage, especially in its proliferating population (Beck et al., 1995). Detailed analysis of mutant embryos lacking zygotic *Cdx2* showed that they do form blastocyst-like structures with TE-like cells (Strumpf et al., 2005). However, these mutant blastocysts never implant due to the abnormality of these

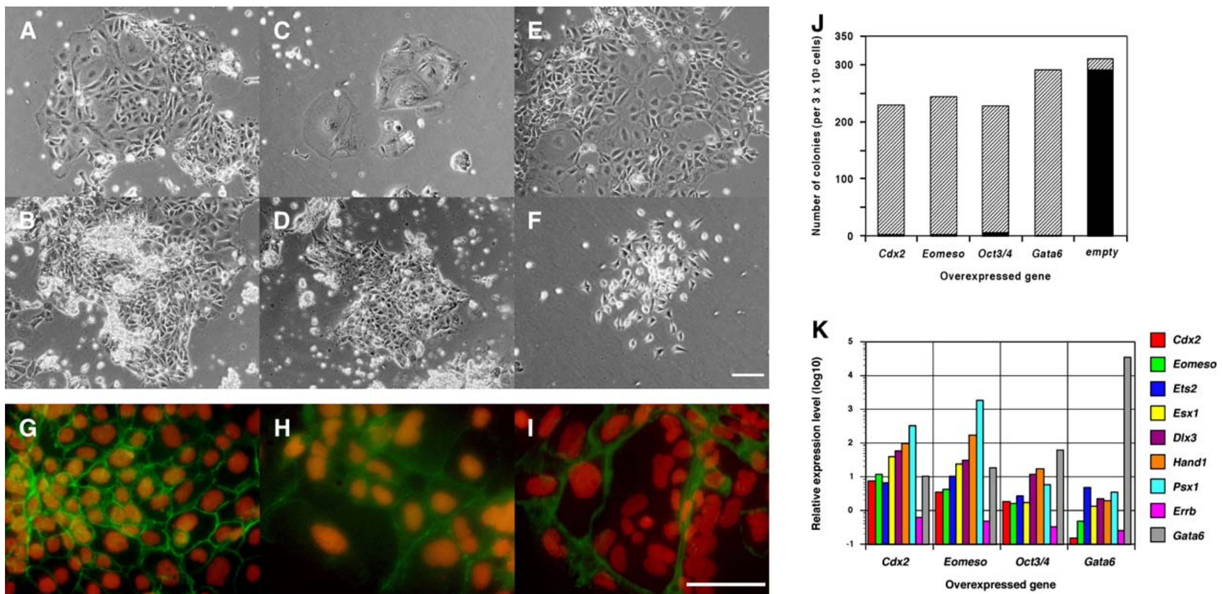

Figure 1. Overexpression of *Cdx2* in ES Cells

(A–F) Photomicrographs of colonies at 6 days after transfection of MGZ5 ES cells with the expression vectors of *Cdx2* (A and B), *Eomeso* (C and D), *Oct3/4* (E), and *Gata6* (F). FGF4 and MEFc were added in (B) and (D) to allow the growth of TS cells. Scale bar is 100 μm.

(G–I) Immunostaining of TE cells generated by overexpression of *Cdx2* (G) and *Eomeso* (H) in MGZ5 ES cells or repression of *Oct3/4* in ZHBTc4 ES cells (I). Cdh3 expression was detected with anti-Cdh3 antibody (green), and nuclei were stained with propidium iodite (PI; red). Scale bar is 100 μm.

(J) Efficiency of differentiation induced by supertransfection. 3×10^3 of MGZ5 ES cells transfected with the transcription factors were selected by puromycin for 6 days, and the numbers of the resulting colonies were scored as their morphological characters. Bars represent the numbers of stem cell colonies (filled) and differentiated ones (hatched).

(K) QPCR analysis of gene expression in the supertransfectants. Total RNA was prepared from each pool of colonies at 6 days after transfection, and the amounts of each transcript were estimated by QPCR. These data were normalized by the amount of *Gapdh* and plotted in logarithmic ratio against the expression level in MGZ5 ES cells, set as 0.

TE-like cells, which ectopically express genes characteristic of pluripotent stem cells, such as *Oct3/4* and *Nanog*, but lack the expression of TE marker genes. These data clearly indicated the importance of *Cdx2* in TE, but it has yet to be revealed whether the function of this gene is essential for initiating the differentiation of TE or for its functional maturation.

In this report, we used an in vitro system of the mouse ES cells for modeling the differentiation to TE to determine the functions of *Cdx2* in TE differentiation and maintenance; we found that the activation of Cdx2 is sufficient to induce differentiation toward the TE lineage. We also show a pivotal role for the interaction between Oct3/4 and Cdx2 in both the establishment and maintenance of the TE lineage.

RESULTS

Overexpression of *Cdx2* in ES Cells Induces Trophectoderm Differentiation

Using an episomal expression vector system, we found that overexpression of *Cdx2* directed morphological differentiation to TE similar to that induced by *Oct3/4* repression (Figure 1A) but distinct from that induced by *Oct3/4* (Figure 1E) or *Gata6* overexpression (Figure 1F; Fujikura et al., 2002). The efficiencies of induction of differentiation were comparable in these three genes (Figure 1J), indicating that these events were not an anomalous effect limited to a subset of transfectants. These cells showed enlarged or multiple nuclei and surface expression of TE marker Cadherin3 (Cdh3, also known as placental cadherin) (Figures 1G and 1I), which were never detected on differentiated cells induced by *Oct3/4* or *Gata6* (data not shown). When the transfectants were selected in the presence of fibroblast growth factor 4 (FGF4), heparin, and mouse embryonic fibroblast (MEF)-conditioned medium, the transfectants expressing *Cdx2* gave rise to trophoblast stem (TS)-like epithelial cells (Figure 1B; Tanaka et al., 1998), suggesting that *Cdx2* is sufficient to trigger differentiation of the TE lineage. Overexpression studies of a second gene, *Eomesodermin* (*Eomeso*), which encodes a T box transcription factor, also induce differentiation as efficiently as *Cdx2* (Figure 1J). The morphology of the resulting cells, which looked quite large and flat in tiny colonies, was slightly different from that induced by *Cdx2* (Figure 1C), but they also expressed Cdh3 (Figure 1H) and formed TS-like colonies in the presence of FGF4, heparin, and MEF-conditioned medium (Figure 1D), indicating that these genes may possess overlapping function in TE differentiation. We also noted with interest that, among the three members of the *Cdx* homeobox gene family in the mouse genome, *Cdx4* is also able to induce differentiation, but overexpression of *Cdx1* had no discernible effect (data not

shown), indicating a diversity of function in ES cells among the members of this gene family.

For our functional analysis, we chose eight transcription factor genes as TE markers, including *Cdx2, Eomeso, Hand1, Esx1, Dlx3, Psx1, Ets2,* and *Errb*; this selection was based on the expression of these genes during TE differentiation and their mutant phenotypes (Rossant and Cross, 2001). Quantitative PCR (QPCR) analyses revealed that all eight TE-associated transcription factors tested were consistently induced by overexpression of either *Cdx2* or *Eomeso* (Figure 1K), suggesting that both *Cdx2* and *Eomeso* represent good candidates in the search for key regulators of TE differentiation.

Inducible Activation of Cdx2 in ES Cells Triggers Proper Differentiation to the TE Lineage

To investigate the differentiation event induced by *Cdx2* in more detail, the expression vector of the 4-hydroxy tamoxifen (Tx)-inducible Cdx2, Cdx2ER, was introduced into ZHBTc4 ES cells, in which both of the endogenous *Oct3/4* alleles are disrupted and a tetracycline (Tc)-regulable *Oct3/4* transgene is expressed to maintain self-renewal (Niwa et al., 2000), resulting in the establishment of the ES cell line 4CER1. These cells could be induced to undergo differentiation into TE in normal culture condition and TS cells on MEF feeder cells in the presence of FGF4 by addition of either Tx (Figures 2A and 2B) or Tc (Figures 2C and 2D), indicating that Cdx2ER functioned properly. Polyploid cells were induced by either Tx or Tc at comparable efficiencies (Figure 2H), but the morphologies of colonies on a gelatin-coated surface were different. On addition of Tc, the colonies mainly contained polyploid cells with multiple small nuclei as found in the parental ZHBTc4 ES cells (Figures 1I and 2C), but on addition of Tx to activate Cdx2ER, the colonies had enlarged nuclei, which was also seen as a result of episomal overexpression of *Cdx2* (Figures 1G and 2A). These data suggest either a preferential induction of polytene cells or an inhibitory effect on nuclear division in endoreduplication that is specific to Cdx2, indicating that the differentiation events induced by activation of Cdx2 or repression of *Oct3/4* were similar but not identical.

The TS cells generated by activation of Cdx2ER expressed the same set of TE markers as that induced by repression of *Oct3/4*. Immunostaining revealed that both sets of TS cells expressed TE markers Ets2 and Cytokeratin 7 (Ck7; Potgens et al., 2001). Interestingly, the TS cells induced by Tx continued to express *Oct3/4* from the Tc-regulable transgene (Figure 2G) at a level comparable to that in undifferentiated ES cells, indicating that Oct3/4 did not block the differentiation event induced by Cdx2ER. QPCR analyses showed that the pattern of the gene regulation triggered by Cdx2ER was almost indistinguishable from that induced by *Oct3/4* repression (Figure 2I). As reported previously, *Cdx2* might be subject to autoregulation as endogenous *Cdx2* was induced immediately after activation of Cdx2ER (Figure 2I; Xu et al., 1999). Downregulation of *Cdx2* in differentiated ZHBTc4 ES cells was accompanied by terminal differentiation of TE, as was shown in a previous report that *Cdx2* is downregulated during differentiation of TS cells (Tanaka et al., 1998). We found that *Cdx2* expression level was maintained in ZHBTc4-derived TS cells at the same time point (Figure 5M). Induction of *Eomeso* by activation of Cdx2 occurred as quickly as in repression of *Oct3/4*, suggesting a dual regulation of *Eomeso* by these factors (Figure 2I).

4CER1-derived TS cells maintained a diploid state during self-renewal (data not shown) and underwent differentiation to the placental lineage following either the withdrawal of FGF4 and MEF or the addition of diethylstilbestrol (DES) (Tremblay et al., 2001), as evidenced by both morphological changes (Figures 2E and 2F) and the induction of marker genes such as *Placental-lactogen-1* (*Pl1*) and *Tpbpa* (data not shown).

Placental Contribution of ES-Derived TS Cells Generated by Activation of Cdx2ER

For further characterization of TS cells induced by artificial Cdx2 activation, we introduced expression vectors of the *EGFPCdx2ER* fusion gene and *pCAG-EGFP-IZ* into EB5 ES cells to further characterize the TS cells induced by the experimental activation of Cdx2. The resulting 5ECER4G20 cells can be maintained as ES cells in the absence of Tx and can be induced to differentiate into TS cells by addition of Tx, FGF4, and MEF. When these ES cells were injected into 30 blastocysts, which were subsequently transferred into uteri of pseudopregnant mice, four chimeric embryos were obtained at embryonic day 12.5 when the EGFP-positive 5ECER4G20-derived cells contributed to the embryo proper (Figure 2K). In contrast, when the cells from the same line were induced to differentiate into TS cells and injected into the same number of blastocycts, the GFP-positive cells showed an exclusively placental contribution in two of the four resultant embryos (Figure 2J), as had previously been found in embryo-derived TS cell chimeras (Tanaka et al., 1998). Immunohistochemical analysis revealed that the 5ECER4G20-derived GFP-positive cells were incorporated into the normal tissue architecture of placenta, confirming their ability to function as TS cells (Figures 2L and 2M), demonstrating that the activation of Cdx2 is absolutely sufficient to induce proper differentiation of TS cells from ES cells.

Reciprocal Inhibition of Transcriptional Activities between Cdx2 and Oct3/4

We previously reported that *Oct3/4* plays a pivotal role in maintaining pluripotency in ES cells (Niwa et al., 2000). However, in the differentiation event induced by Cdx2ER, continuous expression of *Oct3/4* is not sufficient to keep ES cells in the undifferentiated state (Figure 2G). Interestingly, when we tested both the expression of ES-cell-specific genes, such as *Nanog* (Chambers et al., 2003; Mitsui et al., 2003) and *Zfp42/Rex1* (Ben-Shushan et al., 1998), and endogenous *Oct3/4* promoter activity as monitored by the *blasticidin S deaminase* gene (*bsd*) integrated into one of the *Oct3/4* alleles of ZHBTc4 ES cells, we found that all of these genes were downregulated, just as was found after repression of *Oct3/4* by Tc (Figure 3A), indicating that both transcription

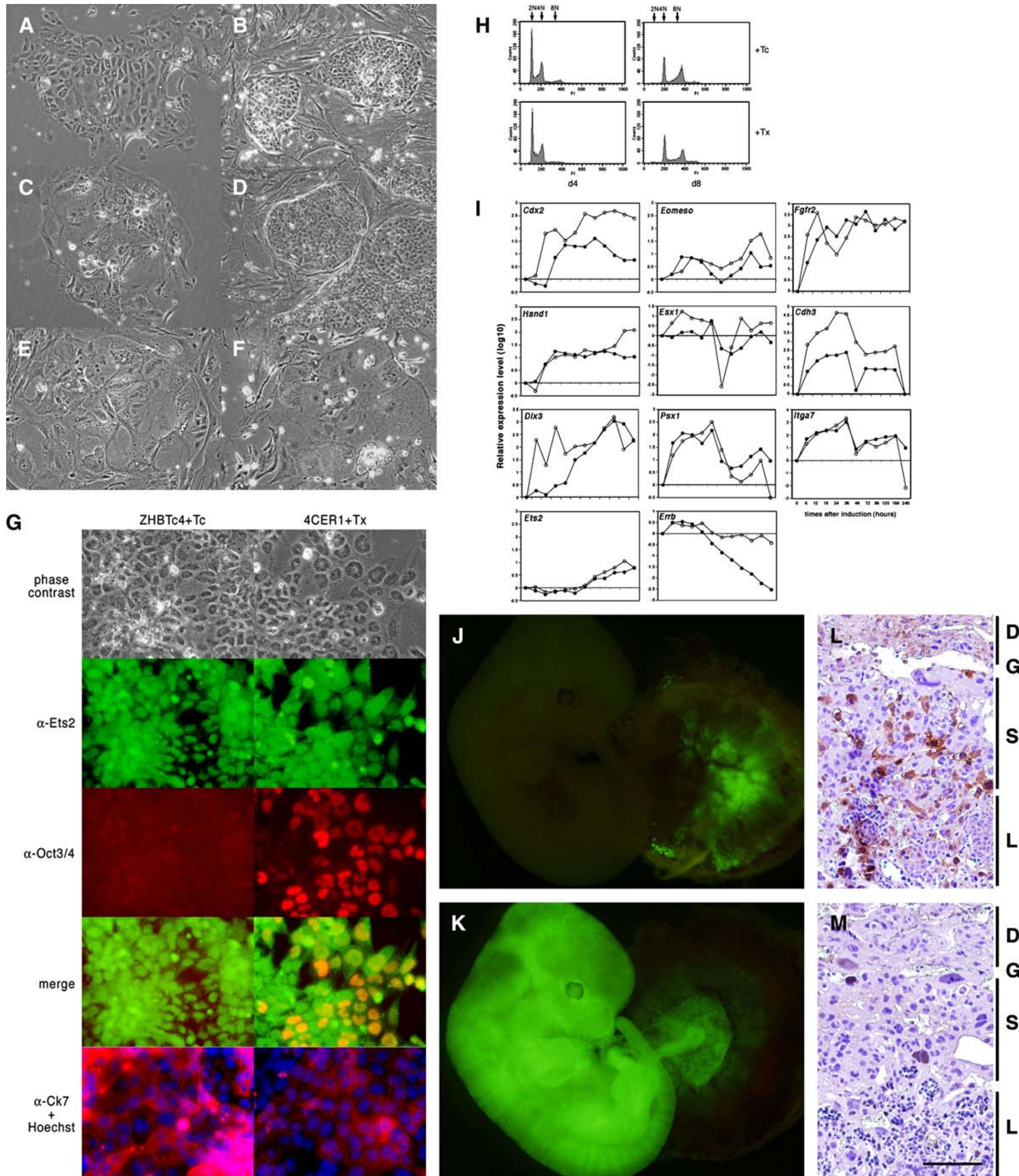

Figure 2. Differentiation of 4CER1 ES Cells to the TE Lineage

(A–F) Photomicrographs of colonies derived from 4CER1 ES cells carrying the *Cdx2ER* transgene. 1×10^3 4CER1 ES cells were seeded in each well of a 6-well plate with Tx (A), Tx, FGF4, and MEF (B), Tc (C), or Tc, FGF4, and MEF (D), followed by culture for 6 days; the resulting colonies were observed by phase-contrast microscopy. Differentiation of TS cells derived from 4CER1 ES cells induced by Tx was induced by withdrawal of Tx, FGF4, and MEF (E) or addition of DES in the presence of Tx, FGF4, and MEF (F).

(G) Marker expression of TS cells. The ZHBTc4-derived TS cells induced by Tc and the 4CER1-derived TS cells induced by Tx were fixed and stained for either Ets2, Oct3/4, or Ck7 with Hoechst 33258 and visualized by fluorescent microscopy.

(H) Analysis of DNA contents of cells stained with PI. 4CER1 ES cells were analyzed at 4 and 8 days with Tc or Tx. Diploid (2N), tetraploid (4N), and octaploid (8N) DNA contents are indicated.

(I) QPCR analyses of the gene expression during differentiation of 4CER1 (open) and ZHBTc4 (filled) ES cells to TE cells induced by Tx. Relative expression levels of each gene were estimated at 0, 6, 12, 18, 24, 36, 48, 72, 96, 120, 168, and 240 hr after addition of Tc. These data were normalized by the amount of *Gapdh* and plotted in logarithmic ratio against the expression level at 0 hr that set at 0.

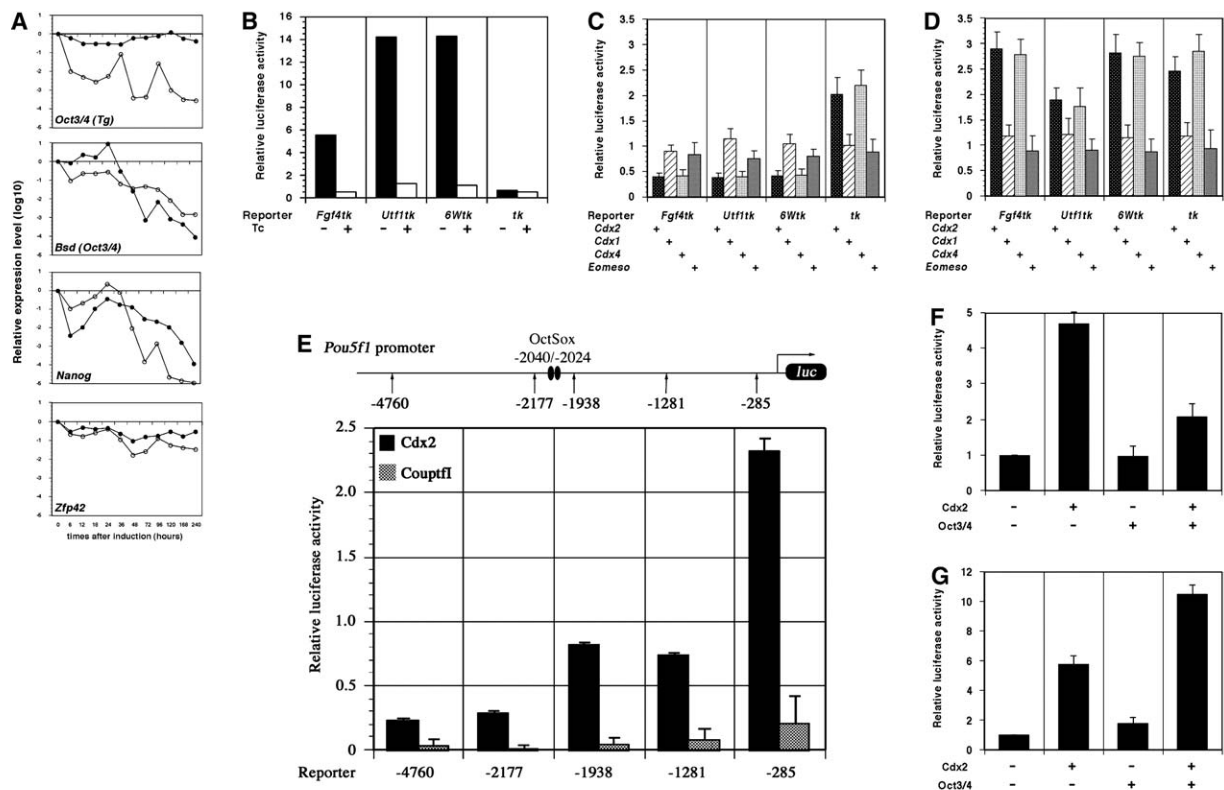

Figure 3. Reciprocal Inhibition between Cdx2 and Oct3/4

(A) QPCR analyses of the stem cell marker gene expression during differentiation of ZHBTc4 ES cells induced by Tc (open) and 4CER1 ES cells induced by Tx (filled) toward TE cells. See the legend for Figure 2I for details.

(B) Luciferase assays of the Oct3/4-dependent reporters in ZHBTc4 ES cells. The Oct3/4-dependent activation of *Fgf4tk*, *Utf1tk,* and *6Wtk* were evaluated as the ratio of the activities in the presence and absence of Tc in ZHBTc4 ES cells. The activity of *tkluc* without Tc was set at 1.0.

(C and D) Cotransfection of the Oct3/4-dependent reporters with the expression vectors for *Cdx1, Cdx2, Cdx4,* or *Eomeso* in the absence (C) or presence (D) of Tc in ZHBTc4 ES cells. The activity of each reporter was normalized against its activity with the empty expression vector *pCAG-IP* (set at 1.0). Data are represented as mean with SEM.

(E) Effect of Cdx2 on autoregulation of *Oct3/4*. Various *Oct3/4luc* reporters were cotransfected with *Cdx2* or *CouptfI* expression vectors into ZHBTc4 ES cells. The reporter activity of each was normalized against its activity with the empty expression vector *pCAG-IP*. Data are represented as mean with SEM.

(F–G) Effect of Oct3/4 on autoregulation of *Cdx2. Cdx2-luc* reporter was cotransfected with *Cdx2* and/or *Oct3/4* expression vector into ZHBTc4 ES cells in the absence (F) and presence (G) of Tc. Data are represented as mean with SEM.

and function of Oct3/4 were inhibited after induction of Cdx2 activity.

To test the possibility that Cdx2 interferes with the transcriptional activator function of Oct3/4, we evaluated the effect of Cdx2 on the activation of the various Oct3/4-dependent reporters in ZHBTc4 ES cells. As shown previously, *luciferase* (*luc*) reporters driven by *Fgf4tk*, *Utf1tk,* and *6Wtk* are activated in ES cells in an Oct3/4-dependent manner (Figure 3B; Niwa et al., 2002). When these reporters were cotransfected with the *Cdx2* expression vector into ES cells, their activity was significantly repressed (Figure 3C). Similar repression was observed by cotransfection with *Cdx4* but not *Cdx1*, indicating that there is a close relationship between the ability to induce differentiation and to block the function of Oct3/4. Interestingly, the reduced *luc* activitity levels of these reporters caused by Cdx2 or Cdx4 were lower than the basal level of the *thymidine kinase* (*tk*) promoter activity, which alone was slightly activated by Cdx2 or Cdx4, suggesting that Cdx2 and Cdx4 actively repress transcription regulated by these Oct3/4-dependent enhancers. When we did the same reporter assay in differentiating ZHBTc4 ES cells cultured for 24 hr with Tc, in which the Oct3/4 protein was depleted, the repressive effect of Cdx2 and Cdx4 was relieved (Figure 3D), suggesting that the repressor function of Cdx2

(J–K) Chimeric embryos derived from 5ECER4G20 ES cells. 5ECER4G20 cells were cultured in the presence of Tx, FGF4, and MEF for 4 days to induce differentiation toward TS cells and then passaged, followed by culture for an additional 4 days. When these TS cells were injected into blastocysts, the embryos developed to chimeras in which GFP-positive cells contributed to placenta (J). In contrast, the same ES-derived cells kept in an undifferentiated state gave rise to embryonic chimeras in the same condition (K).

(L–M) Immunohistochemical analysis of placenta from chimeric embryos J (L) or K (M). GFP-positive cells detected by immunohistochemical staining detected by DAB (brown) were observed in placental tissue only in (L). D, decidua; G, giant cell; S, spongiotrophoblast; L, labyrinth. Bar, 100 μm.

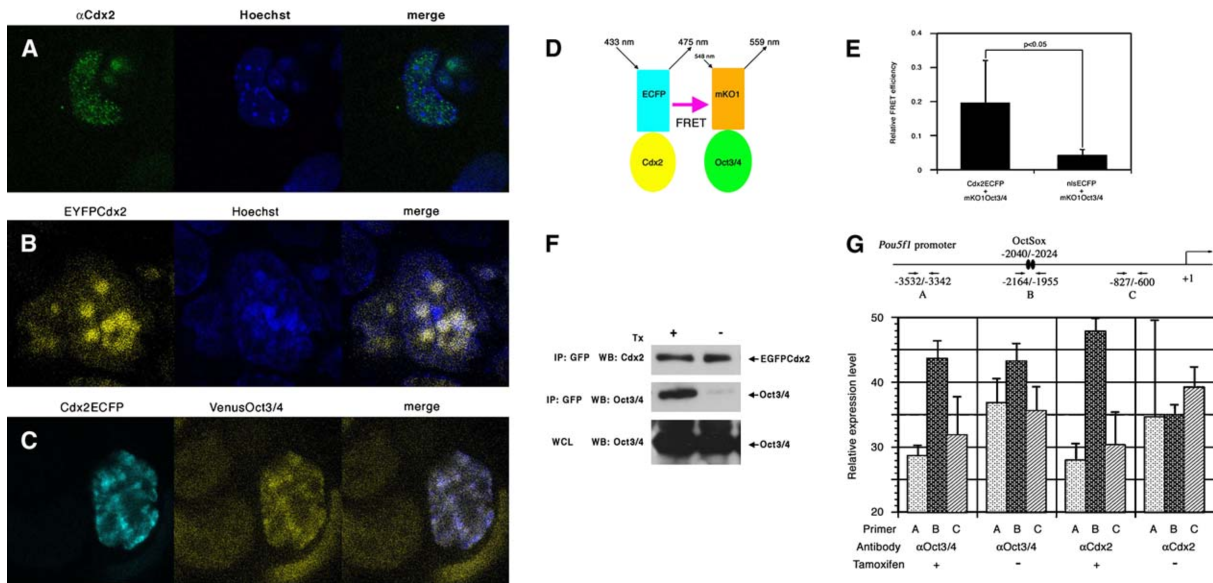

Figure 4. Interaction between Cdx2 and Oct3/4

(A) Localization of Cdx2 in TS cells. TS cell colonies on MEF feeder cells derived from ZHBTc4 ES cells were fixed, stained for Cdx2 (left; green) with Hoechst 33258 (center; blue), and visualized by confocal microscopy. Right panel is merged image.

(B) Localization of Cdx2 in ES cells. ZHBTc4 ES cells were transfected with the *EYFPCdx2* expression vector and fluorescent signals were visualized by confocal microscopy (left) after Hoechst staining (center). Right panel is merged image.

(C) Localization of Cdx2ECFP and Oct3/4 in ES cells. VO-1 ES cells expressing *VenusOct3/4* (middle) were transfected with the *Cdx2ECFP* expression vector (left) and visualized by confocal microscopy. Right panel is merged image.

(D and E) FRET between Cdx2ECFP and mKO1Oct3/4. KOWT7 ES cells expressing *mKO1Oct3/4* were transfected with the *Cdx2ECFP* expression vector and we analyzed a FRET event (D) by confocal microscopy. A statistically significant increase of the FRET signal was detected in nuclei of *Cdx2ECFP* transfectants compared to that in *nlsECFP* transfectants (E: $p < 0.05$ by t test). Data are represented as mean with SEM.

(F) Coimmunoprecipitation of Oct3/4 with Cdx2. When the EGFPCdx2ER protein was immunoprecipitated in the whole-cell lysate (WCL) prepared from 5ECER4 ES cells with an anti-GFP antibody, Oct3/4 protein was coprecipitated when the cells were pretreated with Tx for 24 hr.

(G) ChIP assay for the *Oct3/4* autoregulatory element. Chromatin samples prepared from 5ECER4 ES cells cultured in the presence or absence of Tx for 24 hr were immunoprecipitated with antibodies against either Oct3/4 or Cdx2, and amounts of genomic DNA fragments containing the indicated region of the *Oct3/4* promoter were subjected by QPCR. Data are represented as mean with SEM.

and Cdx4 is dependent on Oct3/4. In contrast, cotransfection of the *Eomeso* expression vector did not show significant effects on these reporter activities (Figures 3C and 3D).

We recently reported that *Oct3/4* gene expression is positively autoregulated in cooperation with Sox2 via an autoregulatory element (ARE) in the distal enhancer (Okumura-Nakanishi et al., 2005). To test the effect of Cdx2 on this autoregulation, we cotransfected various *Oct3/4-luc* reporters with *Cdx2* or *CouptfI*, known repressor of the *Oct3/4* proximal promoter (Ben-Shushan et al., 1995). As shown in Figure 3E, CouptfI repressed all reporter activities as all of *Oct3/4-luc* reporters possessed the *Oct3/4* proximal promoter. In contrast, *Cdx2* had the ability to repress the activities of reporters containing ARE. Taken together, these findings suggest that the repression of Cdx2 was mediated by blockage of Oct3/4 function.

As indicated in Figure 2I, the possibility exists that *Cdx2* is subject to autoregulation in ES cells. We found that the 8.2 kb promoter element of *Cdx2* was activated by Cdx2 in ES cells, as was reported previously in a pancreatic β cell line (Figure 3F; Xu et al., 1999). When the *Oct3/4* expression vector was cotransfected with *Cdx2-luc*, the autoregulation was significantly repressed, whereas *Oct3/4* alone did not show any effect (Figure 3F), indicating that Oct3/4 can block the activity of Cdx2. This repressive effect was observed in an ES-cell-specific manner, as in the case of the effect of Cdx2 on Oct3/4-dependent reporters, since Oct3/4 showed a synergistic effect with Cdx2 to activate the *Cdx2* promoter in differentiated ES cells (Figure 3G) and HeLa cells (data not shown). These data suggested that interaction of Oct3/4 and Cdx2 results in reciprocal repression of their functions in an ES-cell-specific manner.

Cdx2 and Oct3/4 Form a Repressor Complex

How is such reciprocal inhibition between Cdx2 and Oct3/4 achieved? To investigate the molecular mechanism, we monitored intracellular localization of Oct3/4 and Cdx2 by immunostaining and marking with fluorescent tags. In TS cells, endogenous Cdx2 protein was distributed in a speckled pattern in nuclei that did not coincide with high-density Hoechst 33258 fluorescence of heterochromatic regions in interphase cells (Figure 4A). Similar distributions were found for the fusion proteins of Cdx2 with EGFP and its variants (data not shown). However, when *EYFPCdx2* was ectopically expressed in ZHBTc4 ES cells, the fluorescent signals localized in punctuate domains in nuclei, which were distinct

from the localization in TS cells and partially coincided with high Hoechst 33258 fluorescence (Figure 4B), and the similar distributions were found for the wild-type Cdx2 with immunostaining (data not shown). In contrast, homogenous distribution of Oct3/4 in nuclei was observed by both immunostaining (data not shown) and fluorescent tagging (see the two cells on the left without Cdx2ECFP in Figure 4C), but the distribution changed dramatically on introduction of Cdx2. As shown in Figure 4C, in ES cells expressing *Cdx2ECFP*, the VenusOct3/4 fusion protein colocalized with Cdx2ECFP in punctate domains in the nuclei. These data suggest that Cdx2 and Oct3/4 interact with each other and change their localization to transcriptionally inactive regions of the nucleus.

To test the interaction between Cdx2 and Oct3/4 in living cells, fluorescence resonance energy transfer (FRET; Miyawaki et al., 1997) was estimated by confocal microscopic analysis (see Supplemental Data available with this article online). KOWT7 ES cells maintained by expression of the *monomeric version of Kusabira-Orange* (*mKO1*)-Oct3/4 fusion gene were transiently transfected with the *Cdx2ECFP* expression vector, and the FRET signal, indicated as emission of mKO1 by excitation of ECFP, was evaluated in nuclei in which Cdx2ECFP and mKO1Oct3/4 were colocalized (Figure S1A). The signal at 559 nm was confirmed as the FRET signal by the emission scanning with acceptor-bleaching for mKO1Oct3/4 (Figure S1B). In this experimental scheme, a significantly stronger FRET signal was observed in the nuclei in which mKO1Oct3/4 and Cdx2ECFP were colocalized as compared to the nuclei in which mKO1Oct3/4 and nlsECFP were colocalized (Figure 4E). These data indicated that a small moiety of Cdx2 and Oct3/4 in nuclei is present in very close proximity, suggesting possible direct interaction.

To test whether the interaction between Cdx2 and Oct3/4 is indeed direct, we next performed immunoprecipitation analysis using 5ECER4 ES cells expressing the *EGFPCdx2ER* fusion gene. When whole-cell lysate from 5ECER4 ES cells cultured with Tx for 24 hr was immunoprecipitated with an anti-GFP antibody, a significant amount of Oct3/4 was coprecipitated with EGFPCdx2ER (Figure 4F). Since we did not observe coprecipitation of Oct3/4 in cell lysates from either non-Tx-induced 5ECER4 ES cells or 5GER ES cells expressing the EGFPER fusion protein in the presence of Tx (data not shown), we reason that the interaction detected in the lysate form Tx-induced 5ECER4 ES cells occurs between Cdx2 and Oct3/4.

The interaction between Cdx2 and Oct3/4 under physiological conditions was tested by chromatin immunoprecipitation (ChIP) assay (Figure 4G). Chromatin samples of 5ECER4 ES cells cultured in the presence or absence of Tx for 24 hr were immunoprecipitated with anti-Oct3/4 or anti-Cdx2 antibodies, and QPCR was performed using primers that specifically amplify different regions of the *Oct3/4* promoter (Table S2). As result, we found that Cdx2 bound proximately to ARE only in the presence of Tx, whereas Oct3/4 bound to the same element in both the presence and absence of Tx. These data suggest that Cdx2 interacted with Oct3/4 on ARE or bound to a DNA sequence in the immediate region rather than competing for ARE with Oct3/4.

Cdx2 Function Is Dispensable for TE Differentiation

To determine whether *Cdx2* plays an essential role in the differentiation of TE induced by repression of *Oct3/4*, we employed a serial gene-targeting strategy to disrupt both endogenous *Cdx2* alleles in ZHBTc4 ES cells (Figure S2). These ES cells propagate as readily as the parental ZHBTc4 ES cells in the undifferentiated state (Figure 5A). Unexpectedly, however, when Oct3/4 function was inhibited with Tc, both the heterozygous sko113 (*Cdx2*$^{+/-}$) and the homozygous dko23 (*Cdx2*$^{-/-}$) ES cells underwent morphological differentiation into TE that was indistinguishable from that of the parental ZHBTc4 ES cells (Figure 5A). The dko23-derived TE cells expressed Cdh3 (Figure 5B), and FACS analysis confirmed differentiation of polyploid cells from dko23 ES cells following the addition of Tc at efficiencies comparable to those of the parental ZHBTc4 cells (Figure 5C). These data suggested that *Cdx2* is not required for the differentiation of TE induced by downregulation of *Oct3/4*.

The question of the role of *Cdx2* in TS cell formation remained. When the dko23 ES cells were cultured with Tc, FGF4, and MEF, at day 6 they formed the TS-like epithelial cells (data not shown; for a similar result, see Figure 5D), which expressed TE marker Ck7 (Figure 5B). However, these TS-like cells lacking *Cdx2* could not propagate and underwent differentiation after prolonged culture or after passage onto new MEF feeder layers with Tc and FGF4 (data not shown; for a similar result, see Figure 5G), suggesting that *Cdx2* has a unique function in TS cells.

Cdx2 Is Required for Self-Renewal of TS Cells

To confirm the precise function of *Cdx2* in TS cells, we introduced the *Cdx2ER* transgene into dko23 ES cells to obtain ES cell lines in which Cdx2 activity is completely dependent on Tx. One of the resulting cell lines, 23CER1, was confirmed to show the ability to differentiate into TS cells in response to either the downregulation of *Oct3/4* by Tc (Figure 5D) or the activation of Cdx2 by Tx (Figure 5E). During TS cell formation, marker gene expression was induced properly in the absence of *Cdx2* in 23CER1 cells treated with Tc as compared to the expression pattern in parental ZHBTc4 ES cells cultured with Tc (Figure 5M). The 23CER1 cells showed deficient self-renewal of TS cells after differentiation in the presence of Tc and FGF4 on MEF (Figure 5G). However, when the 23CER1-derived TS cells induced by Tc were replated with FGF4 and MEF in the presence of both Tc and Tx, they continued self-renewal (Figure 5H), indicating that the activation of Cdx2ER complemented the function of *Cdx2* to maintain self-renewal in *Cdx2* null TS cells. These cells undergo differentiation upon withdrawal of Tx to inactivate Cdx2ER (Figure 5J) as well as upon removal of FGF4 and MEF (Figure 5K) or addition of DES (Figure 5L), as shown by the upregulation of differentiation marker genes, such as *Tpbpa* and *Pl1*, and downregulation of *Eomeso* (Figure 5N). Continuous propagation of small epithelial cells was observed in the presence of Tx, FGF4, and MEF at least for one month (data not shown). This set of findings indicates that continuous *Cdx2* function is essential for TS cell propagation.

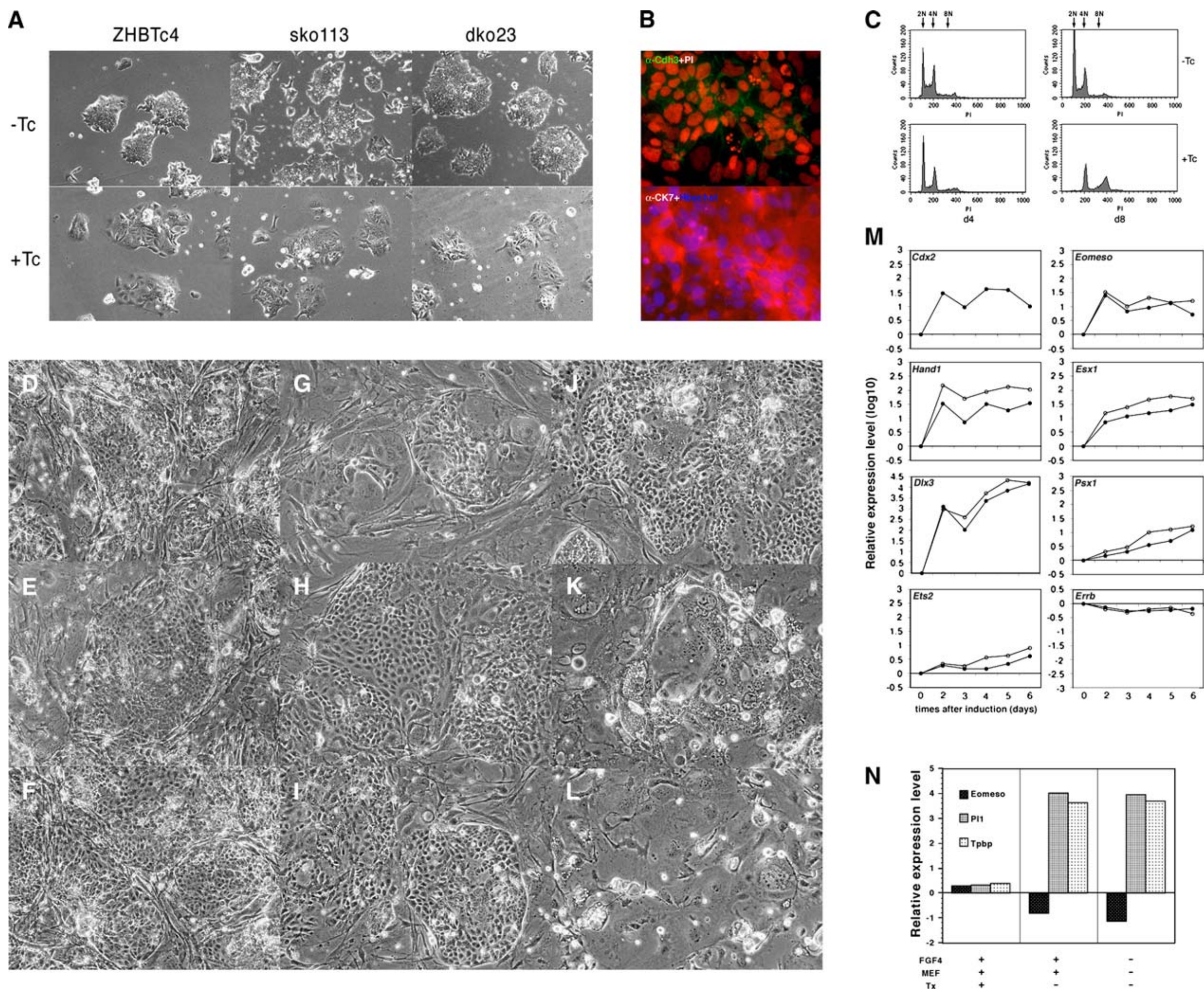

Figure 5. Analyses of *Cdx2* Null ES Cells

(A) Photomicrographs of undifferentiated (top) and differentiated (bottom) sko113 and dko23 ES cells. These cells were cultured with or without Tc for 6 days.

(B) Immunostaining of TE and TS cells derived from dko23 ES cells. Cdh3 expression was detected with anti-Cdh3 antibody (green), and nuclei were stained with PI (red) in TE cells generated from dko23ES cells by addition of Tc (upper panel). The dko23-derived TS cells induced by Tc, FGF4, and MEF were stained for Ck7 with Hoechst 33258 (lower panel).

(C) FACS analysis of DNA contents of dko23 cells stained with PI. dko23 ES cells were analyzed at 4 and 8 days with Tc or Tx. Diploid (2N), tetraploid (4N), and octaploid (8N) DNA contents are indicated.

(D–L) Photomicrographs of colonies derived from *Cdx2* null 23CER1 ES cells carrying the *Cdx2ER* transgene. 23CER1 ES cells were seeded in each well of a 6-well plate with Tc (D), Tx (E), or both Tc and Tx (F) in the presence of FGF4 and MEF, followed by culture for 6 days. Then the TS cells induced by Tc alone were replated into the culture with Tc alone (G) or Tc and Tx (H) in the presence of FGF4 and MEF. These TS cells did not self-renew in the absence of the activated Cdx2ER. When the TS cells induced by Tc and Tx were replated, they formed TS cell colonies in the presence of FGF4 and MEF with Tc and Tx (I) but formed differentiated colonies on withdrawal of Tx (J) or FGF4 and MEF in addition to Tx (K). They also differentaied on the addition of DES in the presence of FGF4, MEF, and Tx (L).

(M) QPCR analyses of the gene expression during differentiation of 23CER1 (open) and ZHBTc4 (filled) ES cells to TS cells in the presence of Tc, FGF4, and MEF. See the legend for Figure 2I for details.

(N) Expression of TS (*Eomeso*), giant cell (*Pl1*), and spongiotrophoblast (*Tpbp*) marker genes after passage of 23CER1-derived TS cells in various culture conditions.

Eomeso Induces TE Differentiation in the Absence of *Cdx2*

We next asked how TE differentiation could be induced in the absence of *Cdx2* function. As shown in Figure 1, *Eomeso* represents a promising candidate for a gene functionally redundant with *Cdx2*. To test this possibility, we introduced the Tx-inducible form of a chimeric *Eomeso* transgene (*EomesoER*) into ZHBTc4 and dko23-5 ES cells, resulting in the establishment of 4EER3 and 23EER1 ES cells, respectively. These ES cells could be induced to differentiate into TE by the addition of either Tc or Tx (Figures 6A–6D), but morphological differentiation was imperfect, showing no

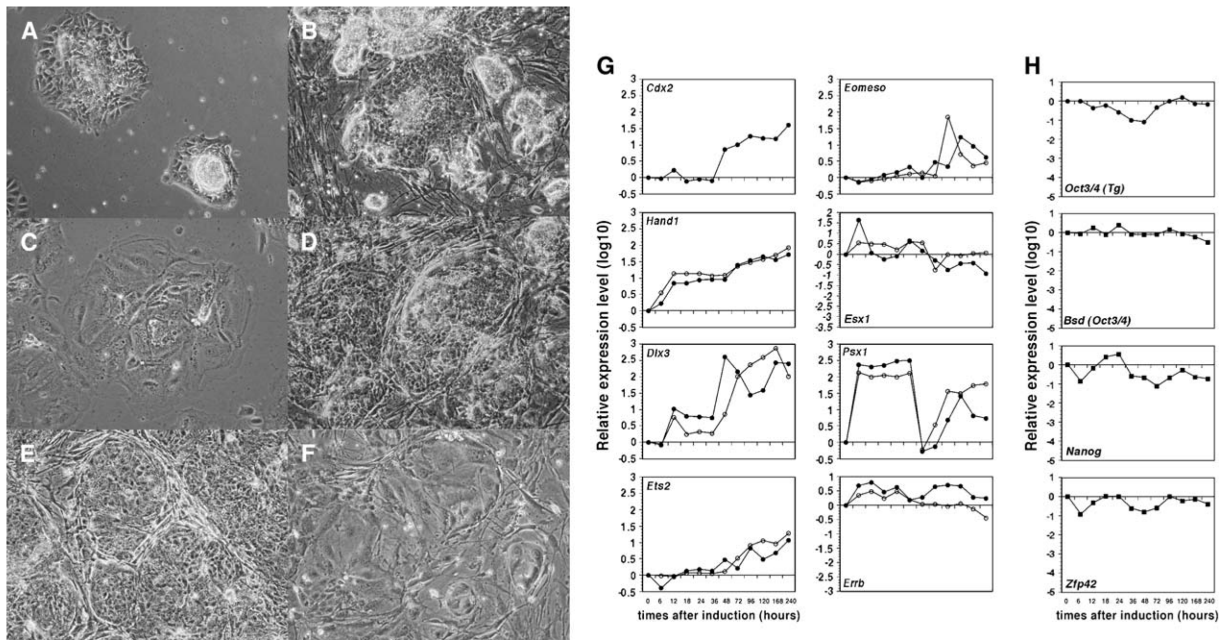

Figure 6. Characterization of Differentiation Event Induced by Activation of Eomeso

(A–D) Photomicrographs of colonies derived from 4EER3 ES cells carrying the *EomesoER* transgene. 1×10^3 4EER3 ES cells were seeded in each well of a 6-well plate with Tx (A), Tx, FGF4, and MEF (B), Tc (C), or Tc, FGF4, and MEF (D), followed by culture for 6 days; the resulting colonies were observed by phase-contrast microscopy.

(E and F) Differentiation of 23EER1 ES cells to TS cells. 23EER1 ES cells were seeded in each well of a 6-well plate with Tc and Tx (E) in the presence of FGF4 and MEF, followed by culture for 6 days. Then the generated TS cells were replated into the culture with Tc and Tx in the presence of FGF4 and MEF (F).

(G) QPCR analyses of the gene expression during differentiation of 4EER3 (filled) and 23EER1 (open) ES cells to TE cells induced by Tx. See the legend for Figure 2I for details.

(H) QPCR analyses of the stem cell marker gene expression during differentiation of 4EER3 ES cells induced by Tx toward TE cells. See the legend for Figure 2I for details.

endoreduplication in Tx-induced TE cells (data not shown). However, gene expression analysis by QPCR revealed that the induction of several TE markers, such as *Hand1*, *Dlx3,* and *Psx1*, occurred as rapidly and at similarly high levels as that induced by downregulation of *Oct3/4* or activation of Cdx2ER (compare Figures 2I and 6G). In contrast, *Cdx2* and *Eomeso* were induced later than 48 hr after addition of Tx (G), indicating that *Cdx2* is not a direct target of Eomeso and that *Eomeso* is not autoregulated. Interestingly, the expression of ES cell markers was not dramatically downregulated (Figure 6H), which correlates well to the results of the reporter assays, in which Eomeso did not inhibit Oct3/4 function (Figure 3C). Both 4EER3 and 23EER1 ES cells differentiated into TS cells in the presence of FGF4 and Tx on MEF, but in the latter case, these TS cells underwent differentiation as had been observed in the parental dKO23 ES cells even in the presence of Tx (Figures 6E and 6F), indicating that loss of *Cdx2* function in TS cells cannot be compensated for by ectopic activation of Eomeso.

Maintenance of *Cdx2* May Work as a Trigger in the Early Embryo

Our data shown above suggested that either downregulation of *Oct3/4* or upregulation of *Cdx2* is able to act as a trigger of TE differentiation. To determine which of these acts in normal development, we performed immunostaining for localization of Cdx2 and Oct3/4 in morula-stage embryos. In the 8-cell-stage embryos, both Oct3/4 and Cdx2 were detected in all nuclei (data not shown). Cdx2 expression starts to decrease in some inner cell nuclei in the early morula (Figure 7A, upper series). In the late morula, Cdx2 was detected only in a subset of the outer cells, whereas Oct3/4 protein was still detectable in all cells (Figure 7A, middle series), and the segregation of the expression domains of these genes was completed in blastocyst (Figure 7A, lower series). The regional loss of Cdx2 expression was first seen in the 10–16-cell-stage embryo and progressed along with development to the 18–22 cell stage, mainly in the inner cells (Figure 7B). These data suggest that loss of Cdx2 in the inner cells in the early morula might be a primary event in the segregation between ICM and TE fates. We suggest that the expression domains of Cdx2 and Oct3/4 may become segregated by mutual inhibition that results in the generation of TE and ICM, which respectively express these genes (Figure 7C).

DISCUSSION

Cell-fate determination is a crucial process of development. Transcriptional regulation by lineage-specific transcription factors is clearly involved in this process. However, little is

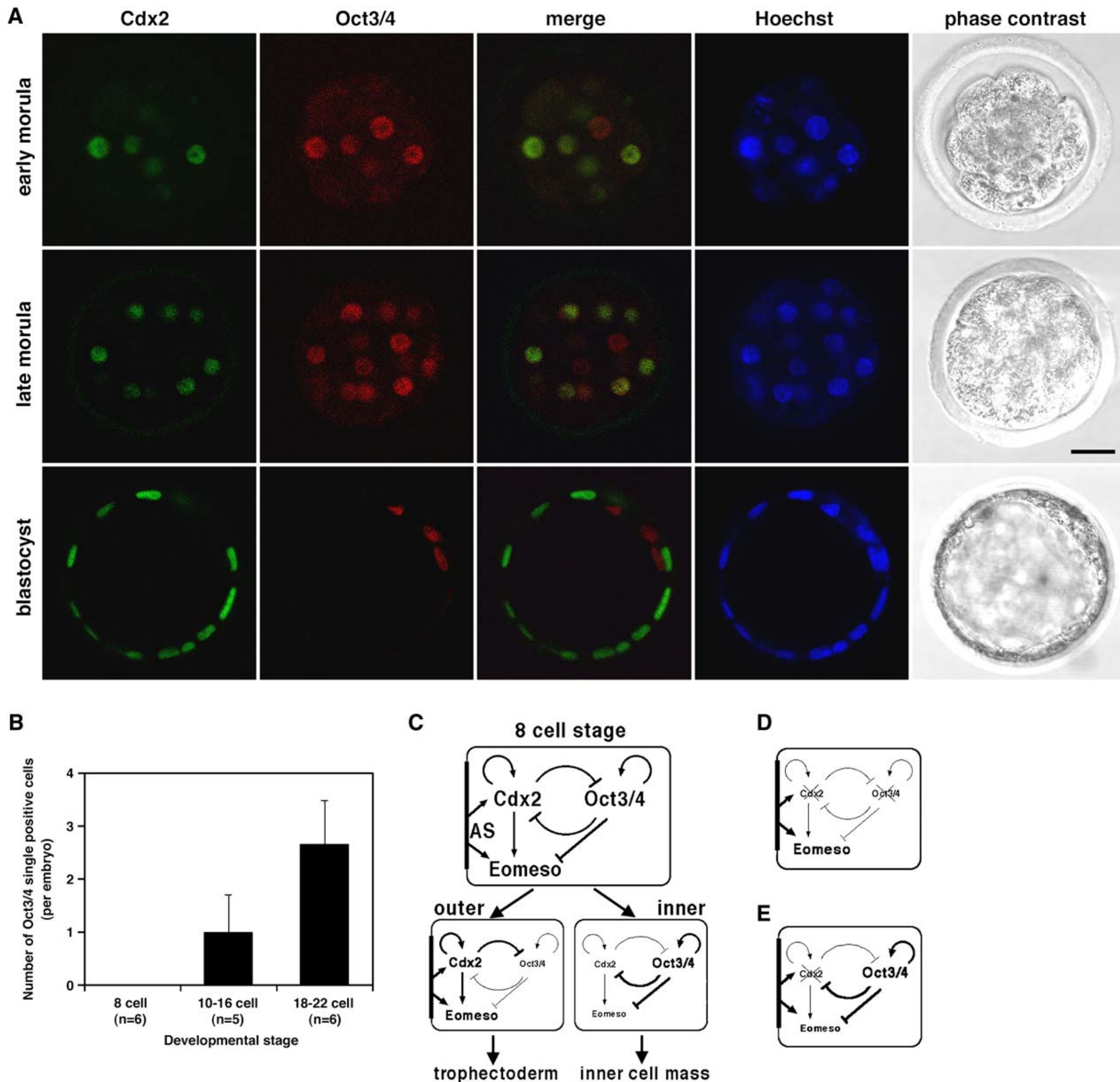

Figure 7. Determination of Trophectoderm in Preimplantation Embryo

(A) Photomicrographs of embryos stained for localization of Cdx2 and Oct3/4. Embryos were fixed, stained for anti-Cdx2 antibody (green), anti-Oct3/4 antibody (red), and Hoechst 33258 (blue), and visualized by confocal microscopy. Merged images for signals of Cdx2 and Oct3/4 and phase-contrast images are also shown. Scale bar is 20 μm.

(B) Number of cells expressing only Oct3/4 per embryo. Data are represented as mean with SEM.

(C–E) Model for segregation of ICM and TE. See Discussion for details.

known about the molecular mechanisms controlling the expression of such factors to trigger proper differentiation at appropriate sites and times. It is a corollary of cell-fate determination that in order for a set of cells differentiate into a new phenotype, a change in gene-expression programs must take place. In early blastocyst-stage embryos, there are only two cell types, pluripotent ICM and differentiated TE, both of which are generated in morula. Here we have shown that the maintenance of Cdx2 activity plays a pivotal role in this process and that reciprocal inhibition between lineage-specific transcription factors, Oct3/4 in pluripotent cells and Cdx2 in trophectoderm, might be involved in the segregation of these cell lineages.

We found that both downregulation of *Oct3/4* and upregulation of *Cdx2* can trigger differentiation of ES cells toward TE, suggesting that the differentiation of TE could be mediated at the level of either transcription or function either by repression or activation of Cdx2 in the inner or outer cells of morula, respectively, or by a converse regulation of Oct3/4. According to previous reports, zygotic *Oct3/4* expression

starts from the 2-cell stage, whereas *Cdx2* expression is upregulated in 8–16-cell stage embryos (Wang et al., 2004). Our immunostaining revealed that Cdx2 expression was initially detectable in both inner and outer cells, when TE fate is being established in the morula, and then Cdx2 expression was specifically maintained in some of the outer cells, although Oct3/4 was expressed in all blastomeres, suggesting that either the activation of *Cdx2* expression in outer cells or its repression in inner cells might be a primary trigger of lineage segregation. It has been reported elsewhere that Oct3/4 protein remains detectable in the TE in bovine blastocysts even after transcription has ceased (Kurosaka et al., 2004), suggesting that the primary function of Cdx2 is to block Oct3/4 function in TE commitment. Since both *Oct3/4* and *Cdx2* can be autoregulated (Xu et al., 1999; Okumura-Nakanishi et al., 2005), their zygotic activation can be achieved automatically from the basal promoter activities for these genes. We speculate that once expression of these genes reaches its threshold levels and an imbalance between Oct3/4 and Cdx2 is created, a reciprocal inhibition system may amplify this asymmetric pattern, resulting in their mutually exclusive expression in two different cell lineages, ICM and TE. This autopoietic scheme is attractive for its great explanatory power in accounting for lineage segregation, given the limited preexisting positional information in morula-stage embryos (Figure 7C). This model can also explain why *Oct3/4* is reexpressed in TE-like cells in *Cdx2* null embryos (Strumpf et al., 2005) and why TE markers are expressed in the inner cells in *Oct3/4* null embryos (Nichols et al., 1998).

Several reports have indicated an inhibitory role of Oct3/4 on TE-specific gene expression. Ezashi et al. reported that Ets2-induced transactivation of the *tau interferon* promoter is repressed by Oct3/4 (Ezashi et al., 2001). In this case, it was shown that the POU domain of Oct3/4 directly interacts with the central domain of Ets2 and acts as a corepressor. In our study, we detected interaction between Cdx2 and Oct3/4 by conventional immunoprecipitation. Some form of interaction was further suggested by the localization of Oct3/4 in nuclei to heterochromatic regions in the presence of Cdx2 and confirmed by FRET in living cells. In addition, ChIP on the *Oct3/4* autoregulatory element revealed that the interaction might occur without competitive binding of Oct3/4 at the target site, suggesting that the complex might act as a repressor on the Oct3/4 targets. However, it should be noted that results from reporter assays suggested a repressor role for the complex of Cdx2 and Oct3/4 on episomal DNA independent of the chromatin context but specific to undifferentiated ES cells. Taken together, these findings lead us to propose that the complex might recruit corepressors expressed in a stem-cell-specific manner to the target site to initiate transcriptional repression and then subsequently recruit them to participate in heterochromatin formation (Thiel et al., 2004). It was recently reported that ectopic expression of *Oct3/4* causes dysplasia in epithelial tissues in adult mice associated with increased transcriptional activity of β-catenin (Hochedlinger et al., 2005). Since *Cdx2* is known as a tumor repressor in intestinal epithelium (Chawengsaksophak et al., 1997) and possesses the ability to repress β-catenin/TCF transcriptional activity (Guo et al., 2004), this phenotype might be the result of the repression of Cdx2 function by ectopically expressed Oct3/4.

How then is the differential regulation of *Cdx2* achieved in the morula? In the late 8-cell stage, the compaction event is followed by the polarization of blastomeres and epithelialization of the outer cells (Johnson and Ziomek, 1981). At the beginning of the compaction event, tight junction components such as ZO-1α and Rab13 are localized apically (Sheth et al., 2000), indicating the establishment of positional information within the blastomere. It is also known that blastomeres are able to divide asymmetrically to form 16-cell-stage embryos with large polarized outer cells and small inner apolar cells (Sutherland et al., 1990). Regulation of *Cdx2* might occur positively or negatively in respect to positional cues. Alternatively, polarization might induce asymmetric distribution of Cdx2 during cell division as found in the case of Ezrin (Louvet et al., 1996). Whatever the case, some as-yet unidentified "apical signal" might work to stimulate Cdx2 autoregulation in the outer cells, resulting in the repression of Oct3/4, whereas any weak residual expression of *Cdx2* in the inner cells could be repressed by colocalized Oct3/4 (Figure 7C).

Although Cdx2 has a strong dominant effect on TE differentiation, its function is not essential for the TE differentiation induced by *Oct3/4* repression. How can this strange discrepancy be explained? We showed that *Eomeso* might have some overlapping function with *Cdx2*. Interestingly, the upregulation of *Eomeso* by repression of *Oct3/4* paralleled that of *Cdx2*, and this was not affected by the extinction of *Cdx2* (Figures 2I and 6M), indicating that *Eomeso* induction does not require *Cdx2*. Activation of Cdx2 in ES cells rapidly induced *Eomeso* expression, but the reverse pattern of induction was not observed (Figures 2I and 6G), suggesting that *Eomeso* might be a target of Cdx2. The evidence that experimentally activated Eomeso can induce TE differentiation in the absence of *Cdx2* (Figures 6A and 6B) also supports this idea. Moreover, *Eomeso* null embryos formed mature TE cells and developed to embryonic day 6.0 with normal levels of *Cdx2* expression (Strumpf et al., 2005), suggesting that loss of *Eomeso* function on TE differentiation can be compensated for by *Cdx2* in the context of TE differentiation. Therefore, although *Eomeso* and *Cdx2* share many functions for TE differentiation and stand similarly under the control of Oct3/4, *Eomeso* seems to function at least partly downstream of *Cdx2*. In *Cdx2* null embryos, morphological TE differentiation was once observed in cells weakly expressing *Eomeso*, although these TE cells subsequently reexpressed *Oct3/4* and underwent apoptosis, indicating that *Cdx2* is not essential for either the initiation of morphological TE differentiation and induction of *Eomeso* in embryos as found in ES cells (Strumpf et al., 2005). We would like here to point out an important difference in the regulation of *Oct3/4* expression between *Cdx2* null embryos and our ES cell system. *Oct3/4* expression is under physiological control in the null embryos, whereas it was experimentally regulated by Tc in our ES cells. Since *Oct3/4* was completely repressed in our ES cell system by Tc independent of Cdx2 function, TE and TS differentiation triggered by repression

of *Oct3/4* could be completed without the Cdx2 function, whereas Oct3/4 is reexpressed in TE in the absence of Cdx2 in the null embryos that might prevent TE differentiation. Therefore, one function unique to Cdx2 is the repression of Oct3/4 expression and function, an activity that Eomeso does not exhibit. We suspected that this is the main reason why *Eomeso* can completely replace the function of *Cdx2* in our ES cell system but not in embryos.

The regulation of *Eomeso* expression and function, however, remains a mystery. Our data suggest that *Eomeso* might lack positive autoregulation (Figure 6G), meaning that a regulatory mechanism to initiate its expression in early embryos should be different from that of *Cdx2*. The lack of appropriate target genes to monitor its activity as well as a good antibody make it difficult to analyze the function at present. We speculate that the putative apical signal (AS) that activates *Cdx2* expression may also stimulate *Eomeso* in the outer cells (Figure 7C). This hypothesis allows us to explain why *Cdx2* null embryos start to generate TE on weak upregulation of *Eomeso* (Figure 7D). We also confirmed that Eomeso does not interfere with Oct3/4 function on its target reporters and mediates rapid downregulation of stem-cell marker genes. In the case of the *EomesoER* transgene introduced using episomal vector system, its strong expression was sufficient to induce complete TE differentiation in the presence of Tx (data not shown), but the weak expression from an integrated copy produced only an imperfect phenotype, suggesting that expression level is critical in this case. Our QPCR analysis revealed that the copy number of *Eomeso* mRNA was ten times higher than that of *Cdx2* in ES cells (Figure S3). These data suggest that Eomeso function might be interfered with by Oct3/4 or other transcription factor(s) in ES cells and embryos (Figure 7C). This second hypothesis may help to explain why *Cdx2* null embryos degenerate at the blastocyst stage, whereas *Cdx2*-null ES cells give mature TE and TS cells in which *Oct3/4* is extinguished (Figures 7D and 7E). Given this set of findings, we suggest that the regulatory interactions between the three transcription factors Cdx2, Oct3/4, and Eomeso are critical to the determination and maintenance of the TE lineage.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

All ES cells were cultured as described previously (Niwa et al., 1998). TS cells are generated and maintained in GMEM supplemented with 10% (v/v) of FCS, 1× sodium pyruvate, 1× nonessential amino acids, 10^{-4} M of 2-ME, 2 μg/ml of sodium heparin (Wako), and 20 ng/ml of recombinant FGF4 (Sigma) on the mitomycin-C treated MEF feeder cells or in the presence of 70% (v/v) of the MEF-conditioned medium. Detailed information about the cell lines and plasmids introduced into them is provided in the Supplemental Data.

Production of Chimeric Embryos

To obtain chimeric embryos, ES and TS cells were injected into C57BL/6J blastocysts, followed by transfer to the uterus of pseudopregnant ICR mice. Embryos were dissected at 12.5 dpc, and fluorescent signals were observed using an Olympus SZX12 fluorescent dissecting microscope and captured with an Olympus DP70 cooled color digital (CCD) camera.

Luciferase Assay

Luciferase assay was done as described previously (Niwa et al., 2002) using the Dual-luciferase assay system (Promega). Please refer to the Supplemental Data for details about the reporter plasmids.

RNA Preparation and Real-Time PCR Analyses

Total RNA was prepared using TRIzol reagent (Invitrogen), as per the manufacture's recommendation. First-strand cDNA was synthesized from 1 μg of total RNA in a 20 μl reaction with oligo dT primer using the ReverTra Ace first-strand synthesis kit (Toyobo). Real-time PCR reaction was done with the iTaq SYBR Green Supermix (Bio-Rad) using the iCycler System (Bio-Rad) or with the iTaq SYBR Green Supermix with Rox (Bio-Rad) using the ABI PRISM7900HT system (Applied Biosystems). The amount of target RNA was determined from the appropriate standard curve and divided by the amount of *Gapdh* mRNA for normalization. Sequences of primers for QPCR were listed in the Table S1. All primer pairs were designed to detect 3′-untranslated regions and endogenous transcript only.

FACS Analysis

Cells were washed with PBS twice and fixed with ice-cold 70% ethanol for 2 hr. After washing with PBS twice, cells were incubated in 0.25 mg/ml of RNaseA in PBS for 30 min at 37ºC and stained with propidium iodide (PI; Molecular Probes) at a final concentration of 50 μg/ml for 30 min at 4ºC. Cell Fluorescence was measured by flow cytometry with FACS Calibur (BD Biosciences).

Immunoprecipitation and ChIP

Whole-cell lysates with TNE buffer (10 mM Tris-Hcl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% NP-40, and 0.2% protease inhibitor cocktail [Nacalai Tesque]) were reacted and precipitated with agarose-conjugated rat anti-GFP monoclonal antibody (MBL), followed by washing five times with TNE buffer. The samples were separated through 12% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and probed with mouse anti-Oct3/4 monoclonal Ab (C-10, Santa Cruz) or mouse antiCdx2 monoclonal Ab (Cdx2-88, BioGenex). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody, and signals were detected with an ECL kit (Amersham).

Chromatin immunoprecipitation was performed with ChIP-IT kit (ACTIVE MOTIF) as described in the manufacturer's protocol. The shared chromatin samples were immunoprecipitated with antiOct3/4 or antiCdx2 antibody, and precipitated DNA was analyzed by QPCR using the primer pairs listed in Table S2.

Immunostaining and FRET Analysis

See Supplemental Data for details.

Supplemental Data

Supplemental Data include three figures, two tables, Experimental Procedures, and References and can be found with this article online at http://www.cell.com/cgi/content/full/123/5/917/DC1/.

ACKNOWLEDGMENTS

This research was supported by a RIKEN grant and grants for the 21st Century COE Program, "Center of Excellence for Signal Transduction Disease: Diabetes Mellitus as Model," from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and the Leading Project (to H.N.). H.N. also received funding from the CREST program of the Japan Science and Technology Agency on the research subject: The High Throughput Creation of Disease Model Cells and the Analysis of Their Function.

Received: November 18, 2004
Revised: May 16, 2005
Accepted: August 29, 2005
Published: December 1, 2005

REFERENCES

Beck, F., Erler, T., Russell, A., and James, R. (1995). Expression of Cdx-2 in the mouse embryo and placenta: possible role in patterning of the extra-embryonic membranes. Dev. Dyn. *204*, 219–227.

Beddington, R.S., and Robertson, E.J. (1989). An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. Development *105*, 733–737.

Ben-Shushan, E., Sharir, H., Pikarsky, E., and Bergman, Y. (1995). A dynamic balance between ARP-1/COUP-TFII, ERA-3/COUP-TFI, and retinoic acid receptor:retinoid X receptor heterodimers regulates Oct-3/4 expression in embryonal carcinoma cells. Mol. Cell. Biol. *15*, 1034–1048.

Ben-Shushan, E., Thompson, J.R., Gudas, L.J., and Bergman, Y. (1998). Rex-1, a gene encoding a transcription factor expressed in the early embryo, is regulated via Oct-3/4 and Oct-6 binding to an octamer site and a novel protein, Rox-1, binding to an adjacent site. Mol. Cell. Biol. *18*, 1866–1878.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell *113*, 643–655.

Chawengsaksophak, K., James, R., Hammond, V.E., Kontgen, F., and Beck, F. (1997). Homeosis and intestinal tumors in Cdx2 mutant mice. Nature *386*, 84–87.

Ezashi, T., Ghosh, D., and Roberts, R.M. (2001). Repression of Ets-2-induced transactivation of the tau interferon promoter by Oct-4. Mol. Cell. Biol. *21*, 7883–7891.

Fleming, T.P. (1987). A quantitative analysis of cell allocation to trophectoderm and inner cell mass in the mouse blastocyst. Dev. Biol. *119*, 520–531.

Fujikura, J., Yamato, E., Yonemura, S., Hosoda, K., Masui, S., Nakao, K., Miyazaki Ji, J., and Niwa, H. (2002). Differentiation of embryonic stem cells is induced by GATA factors. Genes Dev. *16*, 784–789.

Guo, R.-J., Huang, E., Ezaki, T., Patel, N., Sinclair, K., Wu, J., Klein, P., Suh, E.-R., and Lynch, J.P. (2004). Cdx1 inhibits human colon cancer cell proliferation by reducing β-catenin/T-cell factor transcriptional activity. J. Biol. Chem. *279*, 36865–36875.

Hochedlinger, K., Yamada, Y., Beard, C., and Jaenisch, R. (2005). Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. Cell *121*, 465–477.

Johnson, M.H., and Ziomek, C.A. (1981). The foundation of two distinct cell lineages within the mouse morula. Cell *24*, 71–80.

Kurosaka, S., Eckardt, S., and McLaughlin, K.J. (2004). Pluripotent lineage definition in bovine embryos by Oct4 transcript localization. Biol. Reprod. *71*, 1578–1582.

Louvet, S., Aghion, J., Santa-Maria, A., Mangeat, P., and Maro, B. (1996). Ezrin becomes restricted to outer cells following asymmetrical division in the preimplantation mouse embryo. Dev. Biol. *177*, 568–579.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell *113*, 631–642.

Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M., and Tsien, R.Y. (1997). Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin. Nature *388*, 882–887.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Schöler, H., and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell *95*, 379–391.

Niwa, H., Burdon, T., Chambers, I., and Smith, A. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes Dev. *12*, 2048–2060.

Niwa, H., Miyazaki, J., and Smith, A.G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat. Genet. *24*, 372–376.

Niwa, H., Masui, S., Chambers, I., Smith, A.G., and Miyazaki, J. (2002). Phenotypic complementation establishes requirements for specific POU domain and generic transactivation function of Oct-3/4 in embryonic stem cells. Mol. Cell. Biol. *22*, 1526–1536.

Okumura-Nakanishi, S., Saito, M., Niwa, H., and Ishikawa, F. (2005). Oct-3/4 and Sox2 regulate Oct3/4 gene in ES cells. J. Biol. Chem. *280*, 5307–5317.

Pedersen, R., Wu, K., and Batakier, H. (1986). Origin of the inner cell mass in mouse embryos: Cell lineage analysis by microinjection. Dev. Biol. *117*, 581–595.

Potgens, A.J., Gaus, G., Frank, H.G., and Kaufmann, P. (2001). Characterization of trophoblast cell isolations by a modified flow cytometry assay. Placenta *22*, 251–255.

Rossant, J., and Cross, J.C. (2001). Placental development: lessons from mouse mutants. Nat. Rev. Genet. *2*, 538–548.

Sheth, B., Fontaine, J.J., Ponza, E., McCallum, A., Page, A., Citi, S., Louvard, D., Zahraoui, A., and Fleming, T.P. (2000). Differentiation of the epithelial apical junctional complex during mouse preimplantation development: a role for rab13 in the early maturation of the tight junction. Mech. Dev. *97*, 93–104.

Strumpf, D., Mao, C.-A., Yamanaka, Y., Chawengsaksophak, K., Beck, F., and Rossant, J. (2005). Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. Development *132*, 2093–2102.

Sutherland, A.E., Speed, T.P., and Calarco, P.G. (1990). Inner cell allocation in the mouse morula: the role of oriented division during fourth cleavage. Dev. Biol. *137*, 13–25.

Tanaka, S., Kunath, T., Hadjantonakis, A.K., Nagy, A., and Rossant, J. (1998). Promotion of trophoblast stem cell proliferation by FGF4. Science *282*, 2072–2075.

Thiel, G., Lietz, M., and Hohl, M. (2004). How mammalian transcriptional repressors work. Eur. J. Biochem. *271*, 2855–2862.

Tremblay, G.B., Kunath, T., Bergeron, D., Lapointe, L., Champigny, C., Bader, J.A., Rossant, J., and Giguere, V. (2001). Diethylstilbestrol regulates trophoblast stem cell differentiation as a ligand of orphan nuclear receptor ERR beta. Genes Dev. *15*, 833–838.

Wang, Q.T., Piotrowska, K., Ciemerych, M.A., Milenkovic, L., Scott, M.P., Davis, R.W., and Zernicka-Goetz, M. (2004). A genome-wide study of gene activity reveals developmental signaling pathways in the preimplantation mouse embryo. Dev. Cell *6*, 133–144.

Xu, F., Li, H., and Jin, T. (1999). Cell type-specific autoregulation of the Caudal-related homeobox gene Cdx-2/3. J. Biol. Chem. *274*, 34310–34316.