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The *Caudal*-Related Protein Cdx2 Promotes Trophoblast Differentiation of Mouse Embryonic Stem Cells

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ABSTRACT

Besides holding great promise in clinics, embryonic stem (ES) cells represent a valuable tool for studying regulation of early developmental processes, such as cell differentiation in preimplantation embryos. The *caudal*-related homeobox protein Cdx2 is a transcriptional regulator essential for trophoblast lineage, functioning as early as implantation. Using an inducible system, we show that gain of Cdx2 function in ES cells triggers trophoblast-like morphological differentiation, accompanied by ploidy increase, onset of expression of trophoblast-specific markers, and loss of pluripotency-associated gene expression. These data provide an insight into the genetic network that controls lineage specification and functioning in early mammalian development. STEM CELLS *2006;24:139–144*

INTRODUCTION

During mouse preimplantation development, the fertilized oocyte undergoes several successive cleavages to form, by 2.5 days post coitum (dpc), a compacted cluster of approximately 32 cells, called morula. The latter further undergoes spatial reorganization to form the early blastocyst (3.5 dpc) with epithelial-like trophectoderm (TE) cells embedding the blastocoel cavity and inner cell mass (ICM). By the time of implantation (4.5–5 dpc), the ICM differentiates into the epiblast and overlying primitive endoderm (PE). After implantation, the epiblast-contacting TE (polar TE) gives rise to the trophoblast tissues of conceptus, the PE gives rise to the visceral and parietal endoderm, and the epiblast gives rise to the three germ layers of fetus and the extraembryonic mesoderm (reviewed in [1]).

Lineage tracing and transplantation studies provided important clues about the timing of specification of the three blastocyst lineages. PE precursor cells appear to be set aside in the ICM as early as 3.5 dpc [1]. The outer-versus-inner position of cells within late morula and early blastocyst marks prospective TE and epiblast; however, no definitive segregation of the two lineages takes place until the late blastocyst stage, 4.5 dpc [2, 3]. This progressive lineage segregation is coincident with, and in

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some cases shown to be dependent upon, the expression of key lineage-specific transcription factors: Oct4 and Nanog in the epiblast, Cdx2 in the TE, and GATA6 in the PE [1, 4]. A plausible model is that direct genetic interactions between these and other transcriptional regulators, superimposed by the outer-versus-inner positioning within the embryo, play decisive roles in the specification and maintenance of these first lineages.

The *caudal*-related homeobox protein Cdx2 is the earliest trophoblast-specific transcription factor reported to date. The onset of Cdx2 protein expression takes place in the outer cells of the compacted 16-cell morula at approximately 2.5 dpc, thus marking the prospective TE, and persists in both polar and mural TE of 3.5- to 4.5-dpc blastocysts [5, 6]. After implantation, its expression is extinguished in the mural TE but maintained in polar-TE derivatives, such as extraembryonic ectoderm and chorionic ectoderm [7]. An earlier gene-targeting approach has demonstrated that Cdx2 null embryos fail to implant, suggestive of a major defect in TE development [8]. Strumpf et al. [6] recently took a closer look at the possible nature of this defect and concluded that the implantation failure was due to loss of TE epithelial integrity and/or increased incidence of apoptosis of TE. Our analysis of the same Cdx2 mutant strain [8], albeit on a different genetic background, did not show obvious defects in the TE: In most Cdx2 null embryos, the TE segregated from the ICM and maintained its epithelial integrity properly at least until 4.5 dpc and also expressed a typical TE marker, EndoA (unpublished data). In both cases, however, Cdx2 null mutants neither implanted into uteri nor produced TE outgrowths in culture, and derivation of trophoblast stem (TS) thereof repeatedly failed. Thus, though the role of Cdx2 in the initial TE specification remains uncertain, its indispensability for proper functioning and/or maintenance of trophoblast lineage, as early as the time of implantation, is explicit.

ICM-derived embryonic stem (ES) cells have virtually unlimited self-renewal potential in vitro and are referred to as pluripotent due to their unique ability of multilineage differentiation in culture or upon reintroduction into preimplantation embryos [9]. PE and TE derivatives are the two notable exceptions, because they can be generated in culture from ES cells by external cues or populated by ES cells after reintroduction into the preimplantation embryo only at a very low rate [10]. This is likely to reflect the origin of ES cells, the epiblast, which is the lineage that is fully segregated from TE and PE.

This TE/PE-prohibited ES cell–differentiation potential, however, can be diverted by altering gene expression programs. For instance, a forced expression of GATA4 or GATA6, overexpression of Oct4, and genetic ablation of Nanog function have been shown to promote ES cell differentiation into PE-like cells [11–14]. Conditional loss of Oct4 expression in ES cells triggers trophoblast-like differentiation [11], and in agreement with this observation, earlier studies have shown that the ICM of Oct4 null embryos undergoes the same cell-fate switch [15].

In this report, we show that forced expression of the earliest trophoblast-specific regulator Cdx2 can also promote differentiation of ES cells into trophoblast-like cells. We discuss the possible role of Cdx2 and the mechanism of its action in vivo.

Materials and Methods

See Supplementary Information available online.

Results

We have examined whether forced expression of the key trophoblast-specific transcription factor Cdx2 can divert the fate of ES cells. To this end, a transgenic ES cell line that constitutively expressed a fusion between the full-length Cdx2 protein and the mutated ligand-binding domain of the glucorticoid receptor (GR*) was generated (Fig. 1A). Expression of the fusion transcripts was driven by the ubiquitous chicken β-actin promoter and by the cytomegalovirus enhancer (CX). The nuclear translocation of GR*Cdx2 fusion protein could be effectively controlled by adding the synthetic ligand dexamethasone (DEX) to the culture media (Fig. 3C and data not shown). Consistent with the function of Cdx2 as a nuclear transcription factor, we observed no effect of GR*Cdx2 protein which was mainly cytoplasm-confined in the absence of DEX. On the contrary, upon DEX-induced translocation of GR*Cdx2 into the nucleus, the ES cells underwent drastic morphological changes. The growth rate of the cells became slower, and colonies progressively compacted between 0 and 2 days of induction. At day 3, colonies started to spread, firmly attached to the surface of the culture dishes, revealing cells with morphological characteristics distinct from those seen in untreated ES cell cultures (Figs. 1B, 1C).

Differentiated *GR*Cdx2* ES cell colonies seemed to comprise cells of various morphological characteristics. Most remarkably, large flattened cells with polyploid nuclei, a hallmark of TG cells, appeared in these cultures. Such cells were never observed in vehicle (EtOH)–treated *GR*Cdx2* cells (Fig. 1B, left) or in DEX-induced wild-type ES cell cultures (data not shown). Clusters of small compact cells, sometimes persisting even after prolonged (>10 days) exposure to DEX, are likely to represent nondifferentiated ES cells because of the retention of Oct4 expression (Fig. 3C). Their resistance to differentiation can be explained by the failure of DEX to induce nuclear translocation of GR*Cdx2 fusion (Fig. 3C). The reason for this selective resistance was unknown; however, the subpopulation of such ES cells was always relatively minor.

The DEX-induced *GR*Cdx2* ES cell cultures produced cells with increased ploidy (Fig. 2A) and secreted high levels of two mouse placenta steroids, androstenedione and progesterone

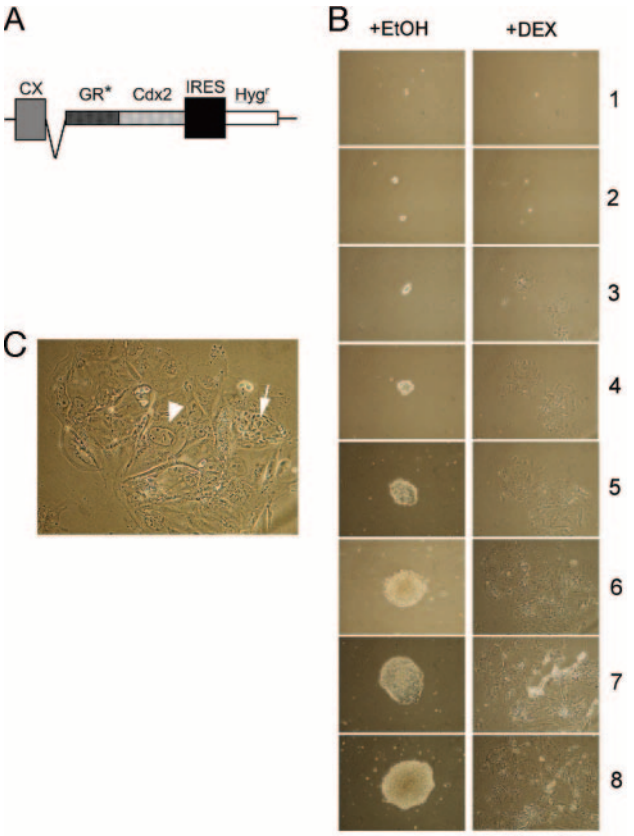

Figure 1. Forced expression of Cdx2 in ES cells induces their differentiation. **(A):** The bicistronic DNA construct used to generate stably transfected transgenic *GR*Cdx2* ES cells. The construct directs ubiquitous expression of the fusion between GR* domain and full-length Cdx2 protein from the cytomegalovirus IE enhancer/chicken β-actin promoter, CX (19). **(B):** Time-lapse recording of *GR*Cdx2* ES cells during the course of DEX-induced differentiation (right panel); the left panel shows the same ES cells induced with the vehicle (control). **(C):** Magnified image of the DEX-induced colony at day 6 from **(B)**; arrowhead points to a typical TG cell, and arrow shows under-differentiated cluster of ES cells or trophoblast stem cells. Abbreviations: DEX, dexamethasone; ES, embryonic stem; GR*, glucorticoid receptor; TG, trophoblast giant.

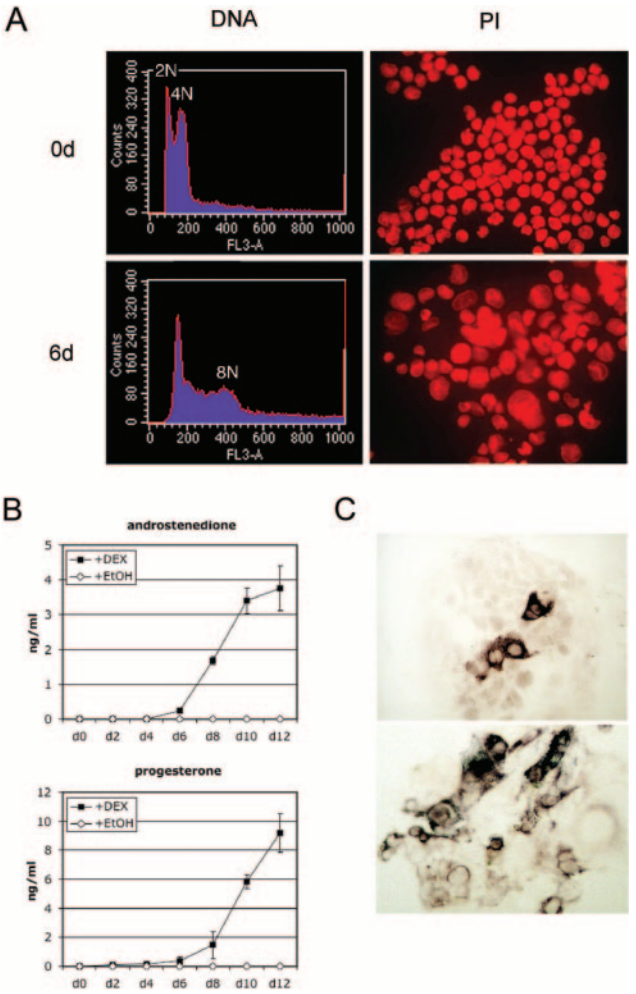

Figure 2. TG characteristics of differentiated *GR*Cdx2* ES cells. **(A):** Flow cytometry analysis of PI-stained *GR*Cdx2* ES cells before (0d) and after 6 days of exposure to DEX (y-axis, cell count; x-axis, PI intensity); 2N (diploid), 4N (tetraploid), and 8N (octaploid) DNA contents; right panels show fluorescent microscopy image of PI-stained cells just before the cytometry analysis. **(B):** Time-lapse analysis of placental steroid in culture media at various time points (0–12 days) of DEX-induced differentiation. **(C):** In situ hybridization with the TG marker Pl-1 antisense RNA of two *GR*Cdx2* ES cell colonies cultured in the presence of DEX for 8 days. Abbreviations: DEX, dexamethasone; ES, embryonic stem; PI, propidium iodide; TG, trophoblast giant.

(Fig. 2B). By day 8 of induction, approximately 80% of colonies showed the onset of RNA expression of the TG marker *placental lactogen-1* (Pl1) in one or more cells (Fig. 2C). The percentage of Pl1-positive cells per total number of cells in a colony (on average, 30%) and levels of Pl1 RNA therein varied (Fig. 2C), possibly reflecting asynchronous differentiation of *GR*Cdx2* ES cells upon DEX treatment. Taken together, these results support the notion that forced expression of Cdx2 protein in ES cells promotes their differentiation into cells with TG characteristics, thus referred to hereafter as TG-like (TGL) cells.

Activation of the leukemia inhibitory factor (LIF)/signal transducer and activator of transcription 3 (STAT3) signaling pathway promotes ES cell self-renewal by suppressing endoderm-like differentiation [9]. However, the withdrawal of LIF during DEX-induced differentiation of *GR*Cdx2* ES cells still resulted in TGL colonies with no sign of enhanced endodermal differentiation (data not shown), suggesting that Cdx2 can override the ES cell self-renewal transcription programs controlled by LIF.

We next surveyed the expression of known lineage markers during the time course of Cdx2-promoted ES cell differentiation. As expected from the ubiquitous activity of the CX promoter, GR*Cdx2 RNA was uniformly present throughout all samples (data not shown). No significant changes in expression

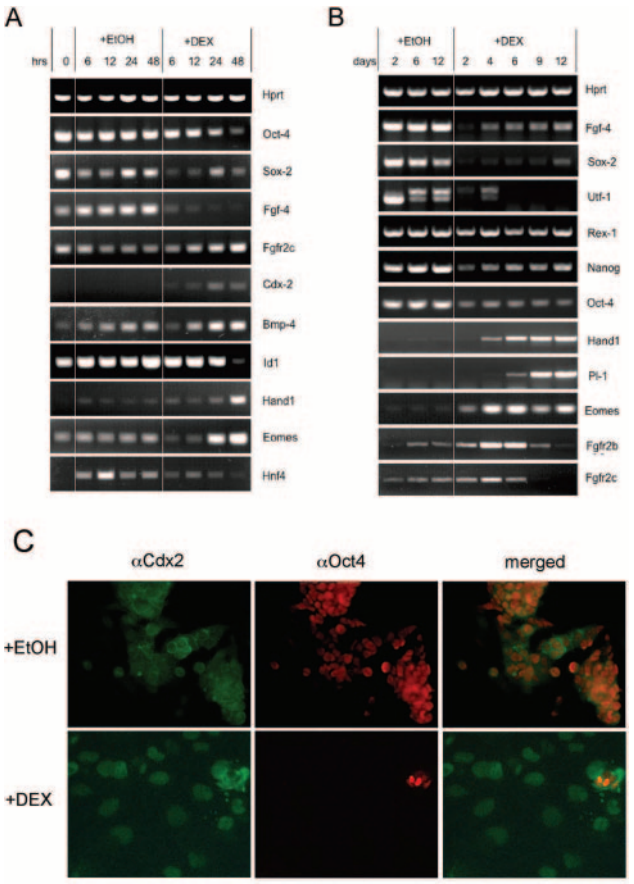

Figure 3. Lineage marker expression analysis during the course of DEX-induced differentiation of *GR*Cdx2* ES cells. Reverse transcription–polymerase chain reaction analysis of short-term **(A)** and long-term cultures **(B)**. **(C):** Double immunolabeling with Cdx2 (green) and Oct4 antibodies (red) of vehicle-treated control (upper row) and 5d-DEX-induced (lower row) cells. Notice that the GR*Cdx2 fusion protein remains cytoplasmic in the cluster of Oct4-positive cells (arrow). Abbreviations: DEX, dexamethasone; GR*, glucorticoid receptor.

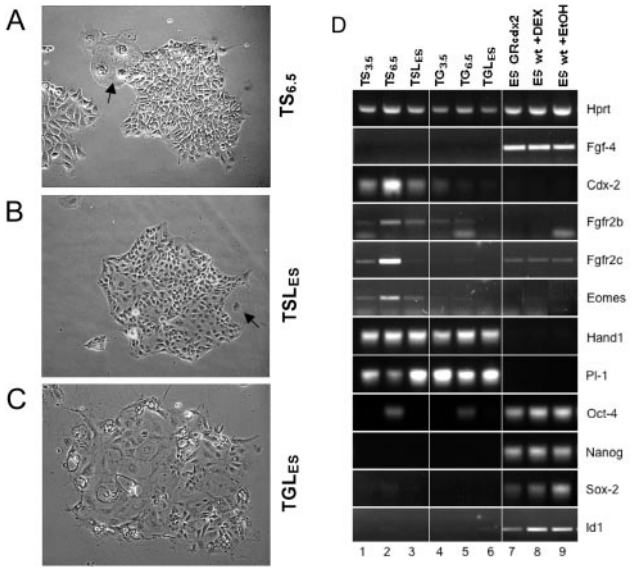

Figure 4. Similar morphology of embryo-derived $TS_{6.5}$ **(A)** and *GR*Cdx2* TSL_{ES} cell colonies **(B)** in the presence Fgf4, heparin, and MEF-CM as per established protocol (18); some TG cells are occasionally seen (arrows). **(C):** TSL_{ES} cell line showed differentiated into TGL_{ES} cells upon the withdrawal of Fgf4 and MEF-CM. Comparison of lineage marker expression in TSL_{ES} and TS cells derived from 3.5-dpc blastocyst ($TS_{3.5}$) and 6.5-dpc ExE ($TS_{6.5}$), using an established protocol (18). Corresponding TG cells differentiated from the TS cells after Fgf4 and MEF-CM withdrawal. Parental *GR*Cdx2* ES cells and wild-type ES cells cultured with (ESwt+DEX) or without (ESwt) DEX stimulation are included as controls. Abbreviations: DEX, dexamethasone; ES, embryonic stem; ExE, extraembryonic ectoderm; GR*, glucorticoid receptor; MEF-CM, mouse embryonic fibroblast–conditioned media; TG, trophoblast giant; TGL_{ES}, ES-derived trophoblast giant–like; TS, trophoblast stem; TSL_{ES}, ES-derived trophoblast stem–like.

levels of most examined marker mRNAs were detected in controls, either DEX-treated wild-type ES cells or vehicle-treated *GR*Cdx2* ES cells (Figs. 3A, 3B; Fig. 4D, lanes 7–9, and data not shown). In contrast, DEX-induced transgenic ES cells displayed remarkable changes in marker gene expression. The pluripotency-associated RNAs Oct4, Sox2, Nanog, Fgf4, and Id1 were rapidly downregulated, as seen in short-term induced cultures (0–48 hours). Expression of these was maintained at low (Oct4) or undetectable (Utf1) levels, yet some genes (Fgf4, Sox2, Nanog) seemed to be slightly upregulated in long-term cultures. However, expression levels were consistently lower than those of noninduced control cells (Fig. 3B). The reason for this might reside in the resistance of a minority of *GR*Cdx2* ES cells to DEX induction (Fig. 3C). Because these cells retain self-renewing capacity, in contrast to mitotically arrested TG cells, their contribution in the total mRNA pool may gradually increase in long-term induced cultures.

Contrary to the pluripotent genes, the pan-trophoblast Hand1 and diploid trophoblast-specific markers Cdx2 (endogenous), Eomes, Fgfr2, and Bmp4 were upregulated, which was already apparent after 48 hours of DEX treatment (Fig. 3A). The induction of endogenous Cdx2 RNA could be due, at least in part, to the positive auto-regulatory loop previously described for this gene [16]. Notably, the expression levels of the diploid trophoblast markers started to decline at day 4–6 of induction, accompanied by an increase of expression of the TG-specific markers Pl1 and Tpbp (4311) (Fig. 3B and data not shown). This observation is consistent with the hypothesis that the DEX-induced transgenic ES cells progress through a diploid trophoblast state that is initially established but cannot be maintained.

Fgf4 and previously unknown substances secreted by mouse embryonic fibroblast (MEF) feeder cells, more recently identified as transforming growth factor–β(TGF-β)/activin [17], support the diploid proliferative state of TS cells, derived from

either 3.5-dpc mouse blastocysts ($TS_{3.5}$) or the extraembryonic ectoderm of 6.5-dpc egg cylinder embryos ($TS_{6.5}$) [18]. Therefore, we hypothesized that the transient, presumably TS-like, state of the differentiating *GR*Cdx2* cells could be maintained upon supply of the same factors. Therefore, the *GR*Cdx2* ES cells were DEX-induced in the presence of Fgf4, heparin, and MEF-conditioned media (MEF-CM), resulting in nearly all colonies acquiring TS-like phenotype after 7 days, with TGL cells also frequently seen. These colonies were individually picked and expanded for 10 days in the presence of DEX. DEX was omitted afterward, and the cells were cultured for at least seven passages (4–7 days each) under TS-permissive culture conditions before being photographed (Fig. 4B). We reasoned that DEX inclusion during the derivation would help to keep the minor population of undifferentiated *GR*Cdx2* ES cells (Fig. 3C) under continuous differentiation-promoting pressure. We also reasoned that without such a pressure, this subpopulation of cells would outgrow relatively slow-proliferating TS-like derivatives during further passaging. Of note, the TS media can support *GR*Cdx2* or wild-type ES cell self-renewal without overt endodermal differentiation, owing to the LIF present in the MEF-CM. The derived, ES cell–free cell lines (Fig. 4B) are referred to hereafter as ES-derived TS-like, or TSL_{ES}, cells due to their unambiguous resemblance to the embryo-derived TS cells (Fig. 4A). As could be anticipated from their TS nature, the withdrawal of Fgf4 and/or MEF-CM resulted in differentiation of TSL_{ES} cells into ES-derived TG-like (TGL_{ES}) cells (Fig. 4C).

We next examined the expression profiles of characteristic marker mRNAs in wild-type and *GR*Cdx2* ES cells, TSL/TGL_{ES} cells, and the embryo-derived TS/TG counterparts (Fig. 4D). *GR*Cdx2* ES cells grown in the presence of vehicle (lane 7), wild-type ES cells induced or noninduced with DEX showed nearly identical expression levels of the pluripotency-associated RNAs, Fgf4, Oct4, Nanog, Sox2, and Id1, as well as low or undetectable levels of trophoblast-specific Cdx2 (endogenous), Fgfr2b/c, Eomes, Hand1, and Pl1 RNAs (lanes 7–9). TSL_{ES} cells exhibited an expression pattern that was most similar to that of $TS_{3.5}$ cells, in respect to expression of the diploid trophoblast markers Cdx2 (endogenous), Fgfr2b, and Eomes, and pan-trophoblast Hand1, as well as to the absence of the pluripotency-associated markers (lanes 1–3). The TG-specific PlI RNA seems to be expressed at a higher level in TSL_{ES} cells (lane 3) than in the embryo-derived counterparts, suggesting a higher incidence of spontaneous TG differentiation in the former cells. $TS_{6.5}$ cells show weak but detectable expression of Oct4 RNA (lanes 2 and 5), which might reflect a contamination with epiblast-derived or unrelated Oct4-expressing cells. Lastly, TGL_{ES}, like $TG_{3.5}$ and $TG_{6.5}$ cells, showed downregulation of TS-specific markers (Cdx2, Fgfr2b), though upregulation of TG-specific PlI was not obvious. We concluded that the ES cell–derived and embryo-derived TS/TG cells share many gene expression characteristics.

DISCUSSION

In this report, we show that forced Cdx2 expression can trigger trophoblast differentiation of mouse ES cells. Whether Cdx2 might play the same instructive role in trophoblast specification during preimplantation development and the possible molecular mechanism of Cdx2 functioning are two points discussed below.

The pluripotency-associated RNAs Oct4, Nanog, Fgf4, and Sox2 are rapidly downregulated in ES cells after Cdx2 induction (Fig. 3). At least two of them, Oct4 and Nanog, are ectopically expressed in the TE of 4.5-dpc Cdx2 null blastocysts ([6] and unpublished data), implying that Cdx2 may represses both genes in vivo. A highly relevant question is whether Cdx2 acts in trophoblast specification through direct repression of *Nanog* and *Oct4*, the key factors necessary for the maintenance of ES-cell/ICM identity, or via independent regulatory circuits. *Nanog* has been postulated to block PE differentiation of pluripotent embryonic cells [13, 14], and thus its repression by Cdx2 is unlikely to account for trophoblast differentiation. The same conclusion is likely to apply to Fgf4, Id1, and Sox2 transcriptional regulators. On the contrary, Oct4 loss by genetic means is sufficient to divert pluripotent ICM and ES cells toward a trophoblast fate [11, 15].

An apparent possibility is that Cdx2-mediated *Oct4* repression in our ES cell model triggers trophoblast differentiation. Therefore, one can anticipate that maintained Oct4 expression in DEX-induced *GR*Cdx2* ES cells should neutralize the effect of Cdx2 and thereby rescue ES cell phenotype. Our preliminary tests suggest that this is indeed the case. We were able to derive DEX-resistant *GR*Cdx2* ES cells by introducing into them a constitutively expressed *Oct4* transgene. Subsequent removal of this transgene restored DEX responsiveness, providing compelling evidence that Cdx2-mediated repression of *Oct4* alone is sufficient for trophoblast differentiation of *GR*Cdx2* ES cells. Moreover, we have observed that Cdx2 is a potent repressor of *Oct4* gene in transient transfection assays and, besides, can be cross-linked to a regulatory region of *Oct4* locus (unpublished data).

There is, however, an observation that argues against a crucial role of Cdx2 in mediating *Oct4* repression in vivo. Oct4 mRNA and protein seem to be present rather uniformly in all cells of the morula, as well as in both ICM and nascent TE cells of the early blastocyst (3.5 dpc). Oct4 protein is visibly downregulated in TE only by the expanded blastocyst stage (4.5 dpc), coincident with the complete segregation of the TE. Cdx2 protein is detectable in the outer cells of the morula from approximately the 16-cell stage onward and, later, in TE ([6] and unpublished data). Therefore, it is coexpressed with Oct4 but

does not repress the latter for at least 1 day. In our ES cell experimental model, the time is sufficient to almost completely suppress *Oct4* transcription (Fig. 3 and data not shown). This raises the possibility that post-translation modifications regulate Cdx2 repressory activity during the morula–to–late blastocyst transition. Alternatively, Cdx2 might interact with the target gene enhancers and/or activatory protein complexes assembled thereon with various affinities, resulting in different kinetics of transcriptional repression in TE. In any case, this Cdx2 activity is anticipated to be less stringent to *Oct4*, as another putative Cdx2 target, *Nanog*, has been shown to be downregulated at the protein level in the TE by the early blastocyst stage [6].

ES cells represent powerful in vitro models for studying regulation of developmental processes. However, these models suffer from the somewhat artificial nature of ES cells and their ex vivo maintenance. Therefore, the conclusion that Cdx2-mediated *Oct4* repression occurs and that this regulatory link is a prerequisite for proper trophoblast development cannot be transferred one-to-one to preimplantation development, but requires careful validation using in vivo models. Genetic experiments, such as double Cdx2/Oct4 null mutants, may help elucidate the molecular control of TE lineage segregation.

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DISCLOSURES

The authors indicated no potential conflicts of interest.

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