Cell Research (2009) 19:1052-1061.

www.nature.com/cr

ORIGINAL ARTICLE

Cross-regulation of the Nanog and Cdx2 promoters

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The first cell fate choice in the mammalian embryo, the segregation of the inner cell mass (ICM) and trophectoderm (TE), is regulated by the mutually antagonistic effects of the transcription factors, Oct4 and Cdx2, while the pluripotency factor, Nanog, is essential to specify the epiblast. We have analyzed the promoters of Nanog and Cdx2, and have found that these two transcription factors are likewise regulated reciprocally. Using an embryonic stem cell line with conditional TE differentiation, we show that Nanog overexpression suppresses the upregulation of TE markers, while Nanog knockdown upregulates the expression of TE markers. We further show that Nanog and Cdx2 bind to and repress each other's promoters. However, whereas Nanog knockout results in detectable *Cdx2* expression in the ICM, we observe no overt disruption of blastocyst development, indicating that Nanog plays a subservient role to Oct4 in segregation of the ICM and TE.

***Keywords*:** Cdx2, Nanog, embryonic stem cells, trophectoderm stem cells

Cell Research (2009) **19**:1052-1061. doi: 10.1038/cr.2009.79; published online 30 June 2009

Introduction

The formation of a multicellular organism from a zygote requires a multitude of steps of cell differentiation. The very first cell fate choice in embryogenesis occurs during the transition from the morula to the blastocyst stage. The resulting two cell lineages, the inner cell mass (ICM) and the trophectoderm (TE), contribute to the embryo proper and to the extra-embryonic tissues of the placenta, respectively. In addition, when cultured *in vitro* under defined conditions, these two cell lineages give rise to two types of stem cells, embryonic stem (ES) cells and trophoblastic stem (TS) cells [1-3]. When injected into blastocysts, ES cells and TS cells retain a memory of their origins, chimerizing the fetus and the placenta, respectively [3, 4]. The ICM and the TE, as well as their *in vitro* counterparts, namely, ES cells and TS cells, express lineage-specific transcription factors, such as Oct4 and Nanog, in the ICM and ES cells, and Cdx2 in the TE and TS cells [5-8]. Oct4 and Cdx2 have been shown to antagonize each other to promote ICM and TE fates [8-10]. Nanog, however, is believed to act at a later stage to specify the epiblast fate, even though Nanog and Oct4 are both involved in maintaining pluripotency and self-renewal in ES cells [5, 7, 11]. In this study, we demonstrate that Nanog can regulate Cdx2 expression, although Nanog plays a subservient role to Oct4 in the segregation of ICM and TE fate.

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Received 6 January 2009; revised 25 March 2009; accepted 11 May 2009; published online 30 June 2009

Results

Reciprocal regulation of Nanog and Cdx2 during ES to TE transition

Our laboratory has established an *in vitro* system to differentiate ES cells into trophectoderm, in which TE differentiation is induced by expression of an activated Ras allele (H-RasQ61L) [12]. We first checked the expression dynamics of lineage-specific transcription factors upon Ras induction by quantitative reverse transcrip-

tion PCR (RT-PCR) analysis (Figure 1A). The induction of Ras leads to enhanced expression of the TE markers, *Cdx2*, *Eomes* and *Hand1*. Surprisingly, *Nanog* is quickly downregulated and *Cdx2* is upregulated within 24 h after adding doxycycline. In contrast, *Oct4* expression levels decline only after 48 h. In addition, western blotting results (Figure 1B) agree with our quantitative RT-PCR analysis: Cdx2 and Nanog are quickly and reciprocally regulated, while suppression of Oct4 occurs more slowly.

Besides iRasES cells, we analyzed the expression of TE and pluripotency markers in iCdx2 ES cells, in which TE differentiation is initiated by Cdx2 [10], instead of H-RasQ61L. Shown in Figures 1C and 1D, the induction of *Cdx2* upregulates *Eomes* and *Hand1*, and suppresses *Nanog* and *Oct4*. Again, *Nanog* is downregulated ahead of *Oct4*. These data imply that Nanog plays a role in antagonizing TE differentiation from ES cells.

To confirm the function of Nanog in TE differentiation from ES cells, we constructed an inducible Nanog (iNanog) ES cell line. Overexpression of Nanog in undifferentiated ES cells suppresses the expression of the TE markers, *Cdx2*, *Eomes* and *Hand1*, at baseline (Figure 1E), suggesting that enforced Nanog expression prevents low levels of transcription from these loci or low levels of spontaneous differentiation in ES cultures. Notably, Oct4 expression is slightly reduced when Nanog is induced, thus ruling out the possibility that Nanog suppresses TE markers by enhancing Oct4 expression (Figures 1E and 1F). When TE differentiation is initiated by transfection of a Ras expression plasmid, induction

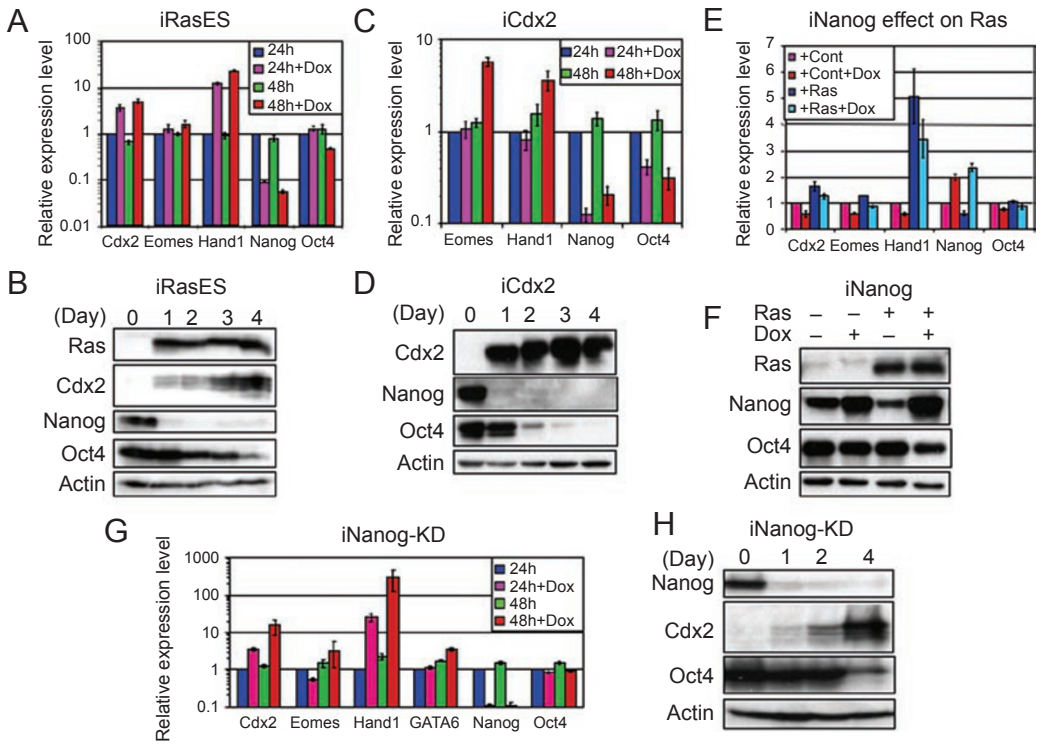

Figure 1 Reciprocal regulation of Nanog and Cdx2 upon *in vitro* trophectoderm differentiation. **(A-D)** Quantitative RT-PCR and western blot analyses of markers of pluripotency and trophectodermal fate in differentiating iRasES **(A** and **B)** and iCdx2 **(C** and **D)** cells. To induce trophectodermal differentiation, iRasES or iCdx2 cells were plated in gelatin-coated tissue culture dishes with TS medium containing 1 μg/ml doxycycline. Cells were collected at indicated time points after adding doxycycline and then subjected to subsequent procedures. Upon Ras induction, quick upregulation of Cdx2 is associated with rapid downregulation of Nanog and relatively slow decrease of Oct4 expression. In iCdx2 cells, induction of Cdx2 also leads to quick suppression of Nanog. Bars indicate mean ± 1σ (n = 3). **(E** and **F)** Nanog overexpression quantitatively reduces the expression of trophectodermal markers. Plasmids expressing GFP or Ras were transfected into iNanog cells; meanwhile, Nanog was induced by adding 1 μg/ml doxycycline. At 48 h after transfection, cells were harvested and analyzed by quantitative RT-PCR **(E)** and western blot **(F)**. Bars indicate mean ± 1σ (n = 3). **(G** and **H)** Knockdown of Nanog leads to trophectoderm differentiation as well as primitive endoderm differentiation. To knockdown Nanog, a modified miRNA targeting the *Nanog* gene is expressed in the doxycycline-inducible system (Supplementary information, Figure S1). Both quantitative RT-PCR **(G)** and western blot **(H)** analyses reveal that upon knockdown of Nanog, Cdx2 is upregulated before Oct4 is significantly affected.

of Nanog reduces the upregulation of *Cdx2*, *Eomes* and *Hand1*. Similarly, in differentiated iRasES and iCdx2 cells, Nanog overexpression quantitatively reduces the expression level of the TE markers, *Cdx2*, *Eomes* and *Hand1* (Supplementary information, Figure S2). Although these data are all consistent with an antagonistic effect of Nanog on TE differentiation, the TE markers are still induced by Ras and Cdx2, implying that both ectopic Ras signaling and Cdx2 expression are qualitatively dominant over Nanog expression in these assays.

Knockdown of Nanog leads to upregulation of TE markers

Previous studies have shown that the knockout or knockdown of Nanog in mES cells results in upregulation of primitive endoderm markers [7, 13], suggesting that Nanog plays a role in the segregation of primitive endoderm from epiblast. In contrast, when Nanog is knocked down in human ES cells, both TE and primitive endodermal markers are upregulated [14]. We wondered whether this discrepancy was due to different biological functions of Nanog in human and mouse ES cells, and thus sought to characterize the effect of Nanog knockdown on mES cell differentiation, especially differentiation into the TE lineage. We engineered an miRNA targeting the *Nanog* gene into the doxycycline-inducible system, resulting in iNanog-KD cells [15]. Adding doxycycline to these cells results in decreased expression of Nanog (Figures 1G and 1H), which was accompanied by increased expression of *Cdx2* and *Hand1*, and the primitive endodermal marker, *Gata6*. *Oct4* expression decreases, but with delayed kinetics. To rule out the possibility of RNAi off-target effects, we knocked down Nanog using two different stealth RNAi oligos [13], which target sequences that are different from the Nanog-targeting miRNA. Again, knockdown of Nanog resulted in an increased expression of both *Cdx2* and *Gata6* (Supplementary information, Figure S3). These knockdown experiments suggest that Nanog suppresses TE differentiation of ES cells *in vitro*.

Nanog and Cdx2 bind to each other's promoter

Niwa *et al.* demonstrated that Oct4 and Cdx2 mutually repress each other by binding to each other's promoters [10]. We asked whether this might also be the case for Nanog and Cdx2. To address this question, we performed ChIP experiments with Nanog and Oct4 antibodies in undifferentiated mES cells, and with Cdx2 antibody in TS cells generated from the iRasES cells [12]. We first looked for binding sites in the *Cdx2* locus. Because an Oct4 binding region has been identified in the intron 1 of *Cdx2* by a ChIP-PET study [16], we scanned not only the *Cdx2* promoter but also the *Cdx2* intron 1 for potential binding sites. We found that Nanog, Oct4 and Cdx2 bind to the promoter and the intron 1 of the *Cdx2* gene (Figure 2). The binding pattern for Nanog is similar to Oct4. Both factors bind to region 4 in the *Cdx2* promoter and to region 7 in the *Cdx2* intron 1. In contrast, Cdx2 binding sites are found in regions 5 and 7.

To further confirm the ChIP results and to define the binding sites for Nanog and Oct4, we performed electrophoretic mobility shift assay (EMSA). A total of 10 DNA segments, ranging from 181 to 272 bp and covering the regions 4 and 7 in the *Cdx2* locus (Supplementary information, Figure S4A), were used as probes in the EMSA. For Nanog EMSA, we could not detect a specific supershift with the Nanog antibody (Bethyl laboratories, A300-397A), regardless of whether mES nuclear extract or purified recombinant Nanog-myc-His protein was used (data no shown). We hypothesized that this Nanog antibody cannot interact with Nanog protein under the conditions of our EMSA assay, and thus instead of detecting the altered mobility of DNA, we applied western blotting to detect altered mobility of the Nanog protein. The modified EMSA showed that the DNA segments, CPa, CPd and CIa, interact with Nanog protein (Supplementary information, Figure S4B). Based on the known consensus Nanog recognition motif (TAAT) [7], we picked several short DNA sequences (38 bp) within the three DNA segments. Nanog binds to two of these short DNA oligos, N1 and N2, from the CPa and CPd regions, respectively (Figure 2E). The DNA oligos, N1 and N2, contains three and two TAAT Nanog recognition motifs, respectively. Mutation of any single TAAT motif does not disrupt the binding of Nanog to the DNA oligos, but when all the motifs are mutated, the binding is abolished. For Oct4 EMSA, specific shifts and supershifts could be detected with the DNA segments CPc and CIe (Supplementary information, Figure S4C). The DNA segments CPa and CIc also have some shifts, which vanish after adding the Oct4 antibody. We then identified two short DNA sequences (38 bp), O1 and O2, containing the Oct4 recognition motif, AT(G/T)(C/T)(A/T)AAT [16], in the CPc and CIe regions. These two DNA oligos interact with Oct4, and the interactions are disrupted when the Oct4 recognition sites are mutated (Figure 2F). The specificity of the interactions was further confirmed by EMSA competitive binding experiments with unlabeled specific and mutated probes (Supplementary information, Figure S4D). These data demonstrate the specific binding sites for Nanog within the Cdx2 promoter and the first intron.

We then investigated the *Nanog* promoter (Figure 3A). Previous studies have shown that Nanog and Oct4 both bind to the *Nanog* promoter in mouse and human ES cells [16, 17], and our ChIP results (Figures 3B and

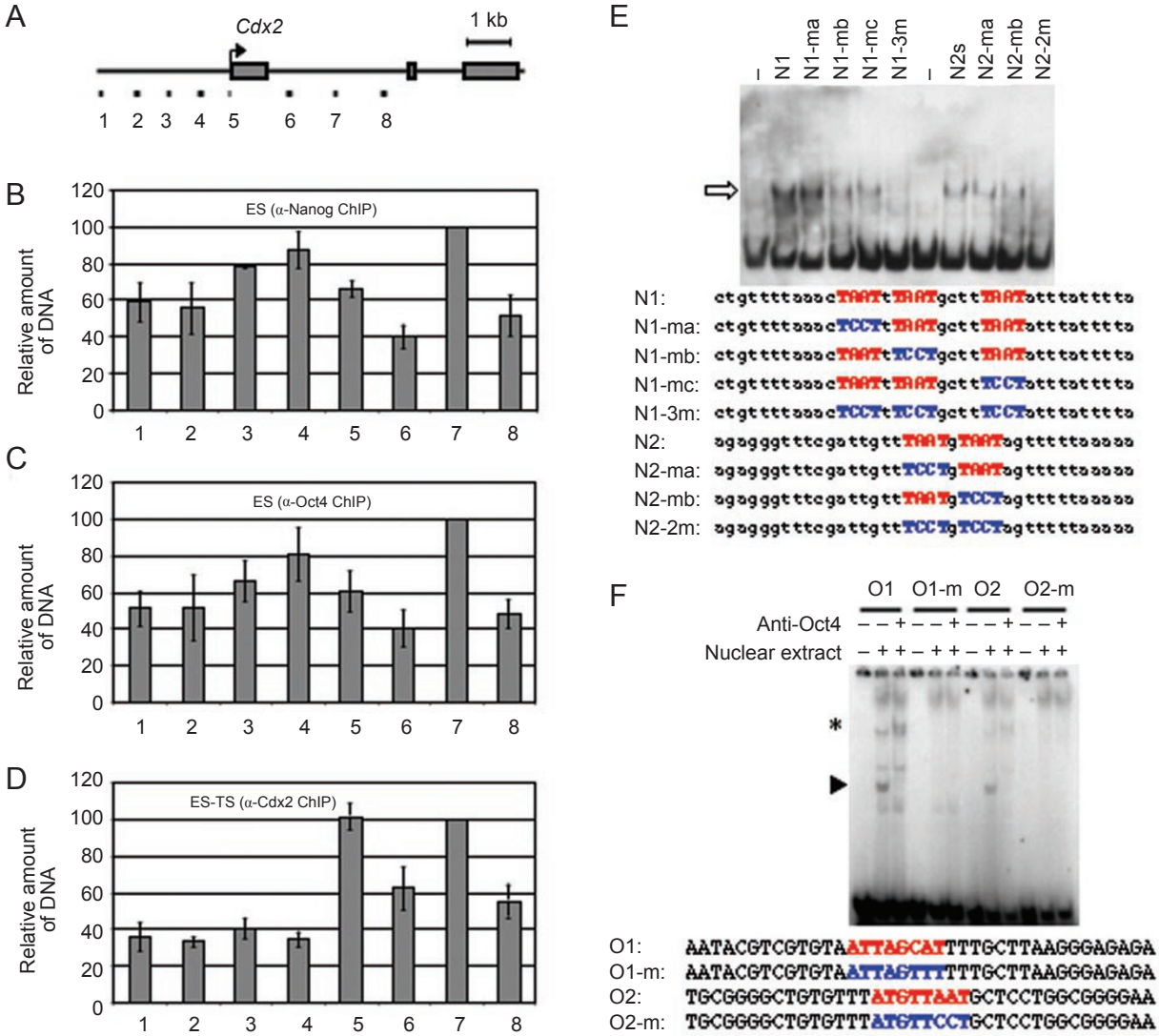

Figure 2 Nanog, Oct4 and Cdx2 bind to the promoter and the intron 1 of the *Cdx2* gene. **(A)** Schematic illustration of the *Cdx2* locus. The rectangles represent three exons of the *Cdx2* gene. The arrow marks the transcription start site. The short bars (labeled 1-8) denote the amplified regions in the real-time PCR analysis. **(B**-**D)** ChIPs were performed with nuclear extracts from undifferentiated iRasES cells **(B** and **C)**, and ES-TS cells **(D)**, and using antibodies recognizing Nanog **(B)**, Oct4 **(C)** and Cdx2 **(D)**. Quantification of the ChIP DNA by real-time PCR was plotted. The relative amount of DNA at region 7 in the *Cdx2* intron 1 was arbitrarily set to 100, and the rest loci were normalized to this region. Bars indicate mean ± 1σ (n = 3). **(E)** EMSA shows that Nanog binds to the Cdx2 promoter. Binding reactions containing 0.5 μg of purified Nanog-myc-HIS protein and 1.5 pmole of DNA probe were run in a 5% TBE polyacrylamide gel. The protein was transferred to a PVDF membrane and Nanog was detected by western blot. DNA segments, N1 and N2, from the *Cdx2* promoter, interact with Nanog and change the electrophoretic mobility of Nanog (marked by an arrow). N1 and N2 have three and two 'TAAT' motifs (shown in red), respectively. Only when all the 'TAAT' motifs are mutated (shown in blue), are the interactions between Nanog and probes disrupted. **(F)** EMSA demonstrates that Oct4 binds to the promoter and to the intron 1 of *Cdx2*. EMSA was performed with nuclear extract from iRasES cells. The radio-labeled probes, O1 and O2, are from the promoter and the intron 1 of *Cdx2*, respectively. The shifts (marked by a triangle) and the supershifts (indicated by an asterisk) are abolished by mutations in the Oct4 recognition sequence (shown in red and blue).

3C) are consistent with those studies. In addition, we found that in TS cells derived following RAS induction in ES cells [12], Cdx2 also binds to the *Nanog* promoter, about 5.5 kb upstream of the transcription start site of the *Nanog* gene (Figure 3D).

We have thus demonstrated that Nanog, Oct4 and

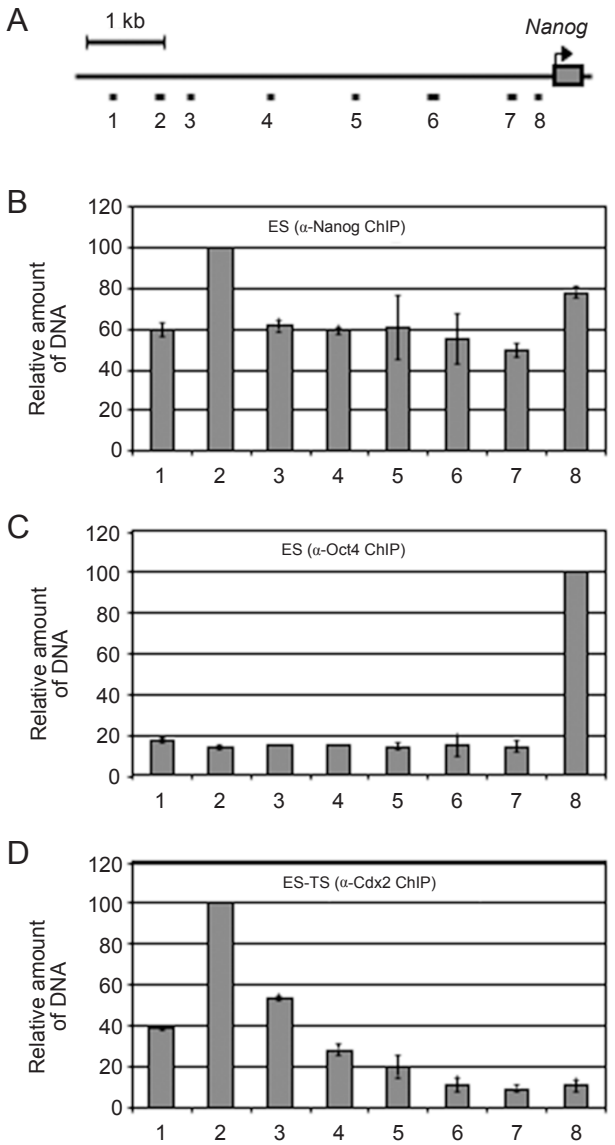

Figure 3 Nanog, Oct4 and Cdx2 bind to the promoter of the *Nanog* gene. **(A)** Schematic illustration of the *Nanog* locus. The rectangle represents the first exon of the *Nanog* gene. The arrow marks the transcription start site. The short bars (labeled 1-8) denote the amplified regions in the real-time PCR analysis. **(B-D)** ChIPs were performed as described in Figure 3. Quantification of the ChIP DNA by real-time PCR was plotted. In each panel, the most enriched region was arbitrarily set to 100 and the rest loci were normalized to these regions. Bars indicate mean ± 1σ (n = 3).

Cdx2 all bind to the *Cdx2* and *Nanog* loci. To determine the regulatory effects of these factors on these genes, we performed luciferase reporter assays. Given that the *Cdx2* intron 1 has binding sites for Oct4, Cdx2 and Nanog, we speculated that it might play a role in transcriptional regulation. Therefore, we constructed a reporter for the *Cdx2* locus that contains the ~3 kb Cdx2 promoter region and the *Cdx2* intron 1 flanking the luciferase gene (pCdx2+CI, Figure 4A). This reporter responds to doxycycline treatment by upregulating the promoter activity (Figure 4B). To assess the effect of Nanog, Cdx2 and Oct4 on the pCdx2+CI reporter, we cotransfected expression plasmids for these three factors along with the reporter plasmid into iRasES cells, with or without doxycycline treatment. In undifferentiated ES cells, Nanog, Oct4 and Cdx2 overexpression suppresses the activity of the pCdx2+CI reporter (Figure 4C, without doxycycline). In differentiated cells (with doxycycline), Cdx2 activates itself, while Nanog and Oct4 do not affect *Cdx2* expression.

We then surveyed the *Nanog* promoter using two reporters (Figure 4D); one (pNanog-L) has a ~6 kb DNA segment from the Nanog promoter, the other (pNanog-S) has a ~3.7 kb Nanog promoter, lacking the distal Nanog binding site and the Cdx2 binding region. The pNanog-L reporter is repressed upon Ras induction, while the pNanog-S is slightly activated, suggesting that the deleted DNA segment contains a Ras-responsive element(s) (Figure 4E). Overexpression of Nanog, Oct4 or Cdx2 represses both reporter activities, except for that the repression of pNanog-S by Cdx2 in differentiated cells is abolished (Figure 4F). This confirms our ChIP results that Cdx2 binds to the deleted region in ES-TS cells. Both Nanog and Oct4 overexpression suppresses the *Nanog* promoter in undifferentiated ES cells, contradicting with the positive feedback regulatory loop among Nanog, Oct4 and Sox2 [17]. Yet, it has been shown that Oct4 suppresses its own promoter when expressed at a high level, while Oct4 activates itself at low expression level [18]. This dosage effect might explain the suppression of the *Nanog* promoter activity by Nanog and Oct4 overexpression.

Nanog and Cdx2 expression in the developing embryo

So far, we have shown that Nanog can modulate TE differentiation from ES cells under artificial conditions of enforced ectopic expression of activated Ras or Cdx2. We thus asked whether Nanog and Cdx2 might cross-regulate each other in the developing embryo. It has been shown that both Nanog and Oct4 are aberrantly expressed in the outer cells of *Cdx2*-null blastocysts [8], suggesting that Cdx2 suppresses not only *Oct4* but also *Nanog* expression in the TE cells. The reciprocal question is whether Nanog suppresses *Cdx2* expression in the ICM. We addressed this by analyzing *Nanog* knockout embryos by immunohistochemistry (IHC). Under identical conditions of embryo staining and confocal imaging, we detected low levels of Cdx2 expression in the

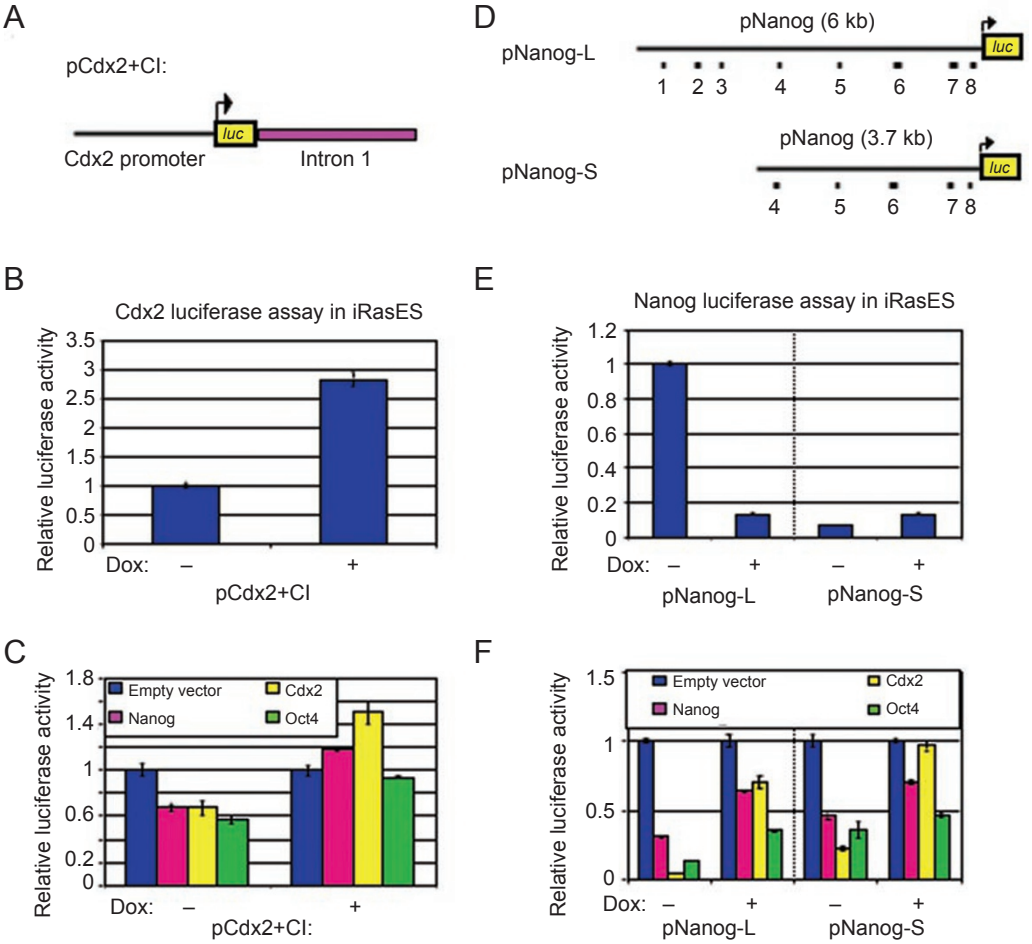

Figure 4 Regulatory effects of Nanog and Cdx2 on each other's expression. (**A**) Schematic illustration of the luciferase reporter for the *Cdx2* locus. The pCdx2+CI reporter has the ~3 kb *Cdx2* promoter region and the intron 1 of *Cdx2* flanking the luciferase gene. (**B**) Ras induction in the iRasES cells enhances the pCdx2+CI reporter activity. This plot showed the result of a representative experiment. Bars indicate mean ± 1σ (*n* = 3). (**C**) The regulatory effect of Nanog, Oct4 and Cdx2 on the *Cdx2* locus. Empty vector, Nanog, Oct4 or Cdx2 overexpression plasmids, together with the luciferase reporter plasmids, were cotransfected into iRasES cells. The transfected cells were cultured in mES medium with or without 1 μg/ml doxycycline. Luciferase activities were measured at 48 h after transfection. In undifferentiated iRasES cells (no doxycycline), overexpression of Nanog, Oct4 and Cdx2, all reduce the transcriptional activity of the pCdx2+CI reporter. In differentiated cells (plus doxycycline), only Cdx2 show positive effect on the reporter. Data from a representative experiment were plotted. Bars indicate mean ± 1σ (*n* = 3). (**D**) Schematic illustration of the two luciferase reporters for the *Nanog* promoter. One (pNanog-L) has an ~6 kb *Nanog* promoter region, and the other (pNanog-S) has a shorter Nanog promoter region (~3.7 kb), lacking the distal Nanog binding site and the Cdx2 binding site. The short bars (1-8) are the same labels as those in Figure 4A. (**E**) The pNanog-L reporter activity is suppressed upon Ras induction, while adding doxycycline does not reduce the pNanog-S activity, implying that the deleted distal promoter region contains the Ras-responsive element(s). Data from a representative experiment were plotted. Bars indicate mean ± 1σ (*n* = 3). (**F**) The regulatory effect of Nanog, Oct4 and Cdx2 on the *Nanog* promoter. The luciferase assays were carried out as described in (**C**). In undifferentiated iRasES cells, overexpression of Nanog, Oct4 and Cdx2, all repress the transcriptional activities of the pNanog-L and pNanog-S reporters. In the differentiated cells, Nanog, Oct4 and Cdx2, also reduce the activities of both reporters, except for that Cdx2 has no effect on the pNanog-S reporter. This is consistent with the fact that the Cdx2 binding region is not included in the 3.7 kb Nanog promoter. The result of a representative experiment was shown. Bars indicate mean ± 1σ (*n* = 3).

Oct4^{+} ICM cells of the *Nanog*$^{-/-}$ embryos, but complete absence of Cdx2 expression in the ICM of *Nanog*$^{+/+}$ or *Nanog*$^{+/-}$ embryos (Figure 5A and Supplementary information, Figure S5). The nuclear expression of Cdx2 remains faint in the ICM of the *Nanog*$^{-/-}$ embryos when compared to the TE, suggesting that the suppressive ef-

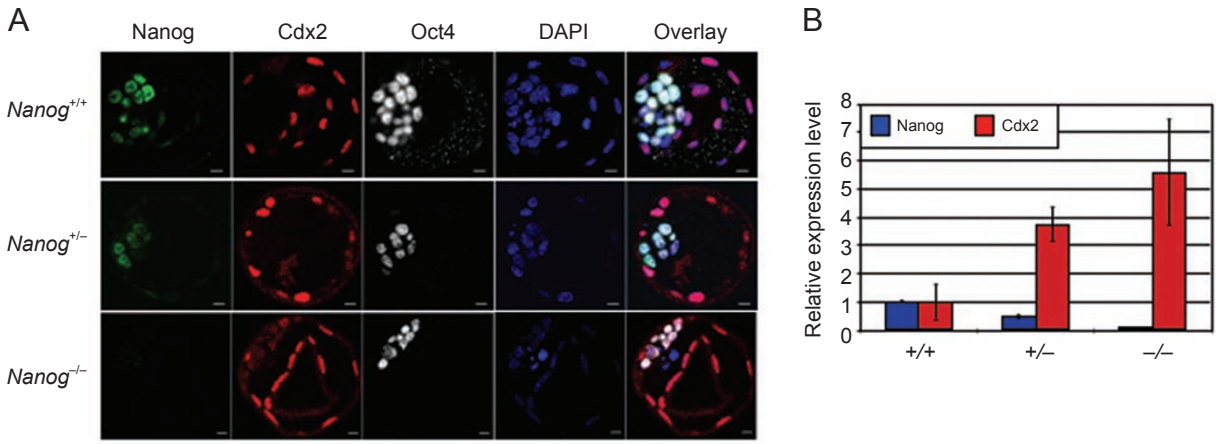

Figure 5 Nanog is necessary to suppress Cdx2 expression in the ICM. **(A)** Loss of Nanog leads to aberrant Cdx2 expression in the ICM. Heterozygous mice carrying a *GFP-IRES-puro* cassette in the endogenous *Nanog* locus were mated to produce a pool of *Nanog*$^{+/+}$ (n = 10), *Nanog*$^{+/-}$ (n = 12) and *Nanog*$^{-/-}$ (n = 6) embryos. *Nanog*$^{+/+}$ embryos were distinguished from *Nanog*$^{+/-}$ and *Nanog*$^{-/-}$ embryos by the absence of GFP expression. These embryos are then subjected to immunofluorescence staining with Nanog, Cdx2 and Oct4 antibodies. Confocal cross-section images are taken under the same setting. Oct4 staining indicates the ICM. Only in the ICM of *Nanog*$^{-/-}$ embryos, Cdx2 expression can be detected. Scale bars: 10 μm. **(B)** *Cdx2* transcription is activated in the ICM of *Nanog*$^{+/-}$ and *Nanog*$^{-/-}$ embryos. Embryos were collected as described in **(A)**. RNA samples from individual immunosurgically isolated ICMs were analyzed by quantitative RT-PCR. *Cdx2* mRNA in the ICM of *Nanog*$^{+/-}$ (n = 16) and *Nanog*$^{-/-}$ (n = 4) embryos is three- to five-fold higher than *Nanog*$^{+/+}$ ICMs (n = 4). *Nanog*$^{+/+}$ embryos were distinguished from *Nanog*$^{+/-}$ and *Nanog*$^{-/-}$ embryos by the absence of GFP expression. *Nanog*$^{-/-}$ embryos were identified based on the *Nanog* mRNA level. Bars indicate mean±1σ.

fects of Oct4 remain dominant. To corroborate the IHC data, we isolated the ICMs of *Nanog*$^{+/+}$, *Nanog*$^{+/-}$ and *Nanog*$^{-/-}$ embryos by immunosurgery, and measured *Cdx2* mRNA levels by quantitative RT-PCR. Consistent with the IHC result, we detected elevated levels of *Cdx2* mRNA in the ICM isolated from *Nanog*$^{-/-}$ embryos compared to *Nanog*$^{+/+}$ embryos. Surprisingly, we also detected elevated Cdx2 mRNA expression in *Nanog*$^{+/-}$ embryos (Figure 5B), which was not apparent on IHC. There are at least two possible explanations for this unexpected result: IHC is less sensitive than quantitative RT-PCR, and thus may not detect low levels of persistent Cdx2 protein in the ICM of *Nanog*$^{+/-}$ embryos. Additionally, Nanog may regulate *Cdx2* expression not only at the transcription level but also through the effects on protein synthesis or degradation. Nevertheless, by both IHC and RT-PCR, we detect elevated levels of Cdx2 expression in the ICM cells of *Nanog*$^{-/-}$ embryos, thus suggesting that Nanog cooperates with Oct4 in Cdx2 repression.

Discussion

Previous studies have established that the segregation of the TE lineage from the ICM is regulated by the mutually antagonistic effects of Oct4 and Cdx2. The internal cells of *Oct4*-null blastocysts divert into the TE lineage [6], and knockdown of *Oct4* in ES cells results in TE differentiation [9]. Niwa *et al.* further demonstrated that Oct4 and Cdx2 not only interact with each other, but also repress each other's expression by binding to each other's promoter [10]. Existing data suggest that Nanog acts at a later stage to antagonize Gata6 and to promote epiblast differentiation. When cultured *in vitro*, the internal cells of *Nanog*-null blastocysts differentiate into parietal endoderm-like cells *in vitro* [7]. Knockout or knockdown of *Nanog* in ES cells leads to primitive endoderm differentiation [7, 13].

Whereas the phenotypic effects of Oct4 and Nanog deficiency argue that Oct4 plays a dominant role in suppressing Cdx2 expression as a means of delineating ICM from TE fate, our results indicate that Nanog and Cdx2 can bind to each other's promoters and influence gene expression. In our *in vitro* model of directed TE differentiation from ES cells, overexpression of Nanog reduces the expression of the TE lineage markers, *Cdx2*, *Eomes* and *Hand1*. Moreover, knockdown of *Nanog* activates not only the primitive endoderm marker, *Gata6*, but also *Cdx2* and other TE markers. A similar observation accompanied shRNA knockdown of *Nanog* in ES cells in another study [19].

Existing ChIP-chip data show that both Nanog and Oct4 occupy the *Cdx2* promoter in human ES cells [17].

Our data demonstrate that like Oct4, Nanog represses *Cdx2* expression by binding to the *Cdx2* locus in mouse ES cells. Reciprocally, Cdx2 binds to and suppresses both *Nanog* and *Oct4* promoters. We have detected elevated transcription of *Cdx2* mRNA and low-level induction of Cdx2 protein in the ICM of *Nanog* knockout embryos, and others have documented that ablation of *Cdx2* leads to aberrant expression of *Nanog* and *Oct4* in trophectodermal cells [8], indicating that the antagonistic effect of Nanog/Oct4 and Cdx2 is not an *in vitro* artifact of cell culture, but instead can be observed in the developing embryo. Nanog might also regulate factors other than Cdx2 that are involved in TE differentiation, as ChIP-chip experiments in human ES cells have shown that Nanog binds to the promoters of *Hand1* and *Eomes* [17]. Although our data show that both Nanog and Oct4 are necessary to completely suppress Cdx2 expression in the ICM, the internal cells commit to the TE lineage in Oct4-null embryos, despite the presence of Nanog, suggesting that the Oct4 role is dominant over that of Nanog in this early fate transition [6]. In our *in vitro* ES cell differentiation system, which allows us to analyze the temporal relationship of Nanog, Oct4 and Cdx2 expression, it appears that Cdx2 upregulation more tightly correlates with Nanog downregulation. Cdx2 protein expression rises even in the face of persistent Oct4 expression, as Oct4 downregulation occurs later (Figure 1B), suggesting that loss of Nanog expression, rather than Oct4, may be the initial molecular event in regulating the Cdx2 promoter. How the signal that mediates the segregation of TE within the compacting morula impinges on the regulation of both Nanog and Oct4 remains a key unanswered question.

Materials and Methods

Cell lines and cell culture

The inducible mouse ES cell lines (Supplementary information, Figure S1) were constructed from the parent cell line, AinV15, as described previously [20, 21]. The iNanog-KD cell line was a gift from Drs Jianlong Wang and Stuart Orkin. Mouse ES cells were cultured in Dulbecco's modified Eagle's medium (Mediatech), supplemented with 15% fetal bovine serum (FBS; Hyclone), 100 μM β-mercaptoethanol (Sigma), 2 mM L-glutamine, 5 000 units/ml penicillin/streptomycin, 0.1 mM MEM non-essential amino acid (Invitrogen), and 1 000 units/ml ESGRO (Chemicon). Mouse TS cells or ES-TS cells were maintained in TS culturing medium; RPMI 1640 (Mediatech), 20% FBS (Hyclone), 100 μM β-mercaptoethanol, 25 ng/ml Fgf4, 1 μg/ml heparin (Sigma), 1mM sodium pyruvate, 2 mM L-glutamine and 5 000 units/ml penicillin/streptomycin (Invitrogen).

Transfection

Transfection of plasmids and Stealth RNAi oligos was performed with Lipofectamine 2000 (invitrogen), as described in manufacturer's instructions. Briefly, 0.8 μg plasmid DNA or 25 pmol Stealth RNAi oligos were transfected into 10^5 cells per well in 24-well culture plates.

RNA purification and reverse transcription

Total RNA was isolated with RNeasy mini kit (Qiagen). RNA from single embryo was purified with Picopure RNA isolation kit (Molecular devices). First-strand cDNA was synthesized with the SuperScript III first-strand synthesis system (Invitrogen). Real-time PCR was performed to quantify the expression levels of genes. Results were normalized with β-actin.

Western immunoblot

Protein extracts, prepared by boiling cell pellets with SDS sample buffer, were resolved by 12.5% SDS-PAGE, followed by transfer onto a PVDF membrane. Membranes were probed with anti-Cdx2 (Biogenex, Mu392-UC), anti-Nanog (Bethyl laboratories, A300-397A), anti-Ras (Upstate, 05-516), anti-Oct4 (Santa Cruz, sc-5279) or with anti-Actin (Abcam, ab3280). Bound primary antibodies were detected by horseradish peroxidase-linked secondary antibodies (GE healthcare), and ECL substrate (GE healthcare), and KODAK BioMax light film.

Chromatin immunoprecipitation

ChIP assay followed a protocol described elsewhere [22]. Briefly, 2×10^8 cells were cross-linked with 1% formaldehyde for 8 min at room temperature. Cells were sonicated at 4 °C, resulting in sheared chromatin with an average size of ~1 kb. Chromatin extract from 5×10^7 cells was used for each IP, with anti-Cdx2 (Biogenex, Mu392-UC), anti-Nanog (Bethyl laboratories, A300-397A) or anti-Oct4 (Santa Cruz, sc-8628). Purified ChIP DNA and input DNA were analyzed by real-time PCR.

Real-time PCR

PCR amplification was carried out in 25 μl reaction volume, containing 12.5 μl of 2× Brilliant SYBR Green QPCR master mix (Stratagene),and 150 nM of each primer. Real-time PCR was performed with the MX3000p machine (Stratagene). PCR cycle parameters were: 10 min at 95 °C, followed by 40 cycles of 30 sec at 95 °C, 30 sec at 57 °C and 30 sec at 72 °C, and then a dissociation curve of the amplified DNA was acquired. See Supplementary information, Data S1 for primer sequences.

Electrophoretic mobility shift assay

Recombinant Nanog-myc-His protein was expressed in BL21, using pRSET expression vector (Invitrogen). The protein was purified with the Probond purification system (Invitrogen), and dialyzed against dialysis buffer (20 mM HEPES, pH 7.6, 20% glycerol, 100 mM KCl, 0.83 mM EDTA, 1.66 mM DTT, protease inhibitor cocktail (Roche)) at 4 °C overnight. Binding reactions were performed at 4 °C for 1 h, in 20 μl mixtures containing 10 mM HEPES (pH 7.6), 10% glycerol, 10 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1.5 pmole 38-bp DNA probe (or 50 ng ~200-bp DNA segment) and 500 ng recombinant Nanog-myc-His protein. The binding mixtures were separated on 5% native PAGE gels and subjected to western blotting.

Mouse ES nuclear extract was used for Oct4 EMSA. Binding reactions were performed at 4 °C for 1 h, in 20 μl mixtures

containing 20 mM HEPES (pH 7.6), 5% glycerol, 50 mM KCl, 1 mM $MgCl_2$, 0.05 mM EDTA, 0.5 mM DTT, 1 μg poly(dI•dC), 0.05% Igepal-CA630, 0.4 pmole ^{32}P-labeled 38-bp probe (or ~200-bp DNA segment) and 1 μg of mES nuclear extract. In supershift experiments, Oct4 antibody (Santa cruz, sc-5279 or sc-8628) was added into the binding reaction without the DNA probe, and the DNA probe was added after 30-min incubation at 4 °C. For EMSA competitive binding experiments, unlabeled DNA was added after the initial incubation for additional 30 min. The binding mixtures were resolved on 5% native PAGE gels. The gel was dried and exposed to a phosphorimage screen (GE healthcare). The image was scanned with the GE healthcare Storm 860 imaging system.

Luciferase assay

Luciferase reporter plasmids were constructed by cloning the promoter or intron DNA segments (Figure 5) into pGL3 vector (Promega). To construct overexpression plasmids, full-length cDNA of mouse Nanog, Oct4 or Cdx2, was inserted into pCAGIPuro. Luciferase reporter plasmid (0.4 μg) and pRV-SV40 (8 ng; Promega), together with 0.4 μg pCAGIPuro empty or overexpression plasmid, were co-transfected into 10^5 cells per well in 24-well culture plates. At 48 h after transfection, luciferase activities were measured with the dual-luciferase reporter assay system (Promega) and the Fluoroskan Ascent FL (Thermo Fisher Scientific). The plotted data in Figure 5 were from a representative experiment with triplicate samples. The experiments were repeated twice, and the data were consistent.

Embryo collection and culture

Nanog heterozygous knockout mice carrying a *GFP-IRES-puro* cassette in the endogenous *Nanog* locus were described elsewhere [23]. *Nanog*$^{+/-}$ females that were superovulated by PMSG and HCG (7.5 IU each, Sigma) were mated with *Nanog*$^{+/-}$ males. Two-cell embryos were collected from oviduct of plugged females, and cultured in KSOM+AA (Millipore MR-121-D) media until the morula or blastocyst stage.

Immunohistochemistry

Embryos were fixed with PBS plus 4% paraformaldehyde for 30 min, permeabilized with PBS plus 0.2% Triton for 30 min, blocked with PBS plus 3% BSA for 2 h and incubated with primary antibodies (1:50 anti-Cdx2 (Biogenex, Mu392-UC), 1:250 anti-Nanog (CosmoBio, REC-RCAB0002P-F) and 1:20 anti-Oct4 (Santa Cruz, sc-8628)) in blocking solution overnight at 4 °C. After three washes with PBS plus 0.1% Tween 20 for 5 min, embryos were incubated with 1:500 dilution of secondary antibodies (Invitrogen, A21441, A21201 and A21447) in PBS for 3 h at 4 °C. Embryos were then washed with PBS and mounted on slide glasses with antifade reagent (Invitrogen, P36930). Confocal images were taken with Zeiss LSM510 confocal microscope in the MRDDRC Imaging Core at Children's Hospital Boston.

Immunosurgery

Blastocysts were treated with 0.5% protease for 5 min to remove zona pellucida, followed by treatment with FHM plus 20% anti-mouse serum for 30 min at 37 °C. After 3 washes with FHM plus 10% FBS, blastocysts were treated with FHM plus 20% guinea pig complement for 30 min at 37 °C. After completely removing trophectodermal cells by pipetting, resultant ICM were subjected to RNA purification.

Acknowledgments

GQD was supported by grants from the National Institutes of Health, the NIH Director's Pioneer Award of the NIH Roadmap for Medical Research, and private funds contributed to the Harvard Stem Cell Institute and the Children's Hospital Stem Cell Program, USA. GQD is a recipient of Clinical Scientist Awards in Translational Research from the Burroughs Wellcome Fund and the Leukemia and Lymphoma Society, and is an Investigator at the Howard Hughes Medical Institute. KH was supported by the NIH Director's Innovator Award and the Harvard Stem Cell Institute, USA.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)