Stem Cell Reports
Report

ISSCR
OPEN ACCESS

OCT4 Coordinates with WNT Signaling to Pre-pattern Chromatin at the *SOX17* Locus during Human ES Cell Differentiation into Definitive Endoderm

Lei Ying,[1] Jason A. Mills,[1] Deborah L. French,[1,2] and Paul Gadue[1,2,*]
[1]Center for Cellular and Molecular Therapeutics, The Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA
[2]Department of Pathology and Laboratory Medicine, The Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA
*Correspondence: gaduep@email.chop.edu
http://dx.doi.org/10.1016/j.stemcr.2015.08.014
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

SUMMARY

We demonstrate that the pluripotency gene *OCT4* has a role in regulating differentiation via Wnt signaling. *OCT4* expression levels in human embryonic stem cells increases transiently during the first 24 hr of in vitro differentiation, with OCT4 occupancy increasing at endoderm regulators such as *SOX17* and *FOXA2*. This increased occupancy correlates with loss of the PRC2 complex and the inhibitory histone mark H3K27me3. Knockdown of OCT4 during differentiation inhibits mesendoderm formation and removal of the H3K27me3 mark from the *SOX17* promoter, suggesting that OCT4 acts to induce removal of the PRC2 complex. Furthermore, OCT4 and β-catenin can be co-immunoprecipitated upon differentiation, and Wnt stimulation is required for the enhanced OCT4 occupancy and loss of the PRC2 complex from the *SOX17* promoter. In conclusion, our study reveals that OCT4, a master regulator of pluripotency, may also collaborate with Wnt signaling to drive endoderm induction by pre-patterning epigenetic markers on endodermal promoters.

INTRODUCTION

In mammals, cells from the blastocyst stage can be used to form embryonic stem (ES) cell lines that can self-renew in culture while maintaining the ability to differentiate into cells of the three germ layers (Reubinoff et al., 2000). Directed in vitro differentiation protocols generally attempt to utilize our knowledge of the normal signaling pathways guiding embryogenesis and mimic this process in vitro, theoretically providing an unlimited supply of any desired cell type (Murry and Keller, 2008). The molecular mechanisms underlying pluripotent stem cell differentiation are of great interest for understanding development, disease, and regenerative medicine.

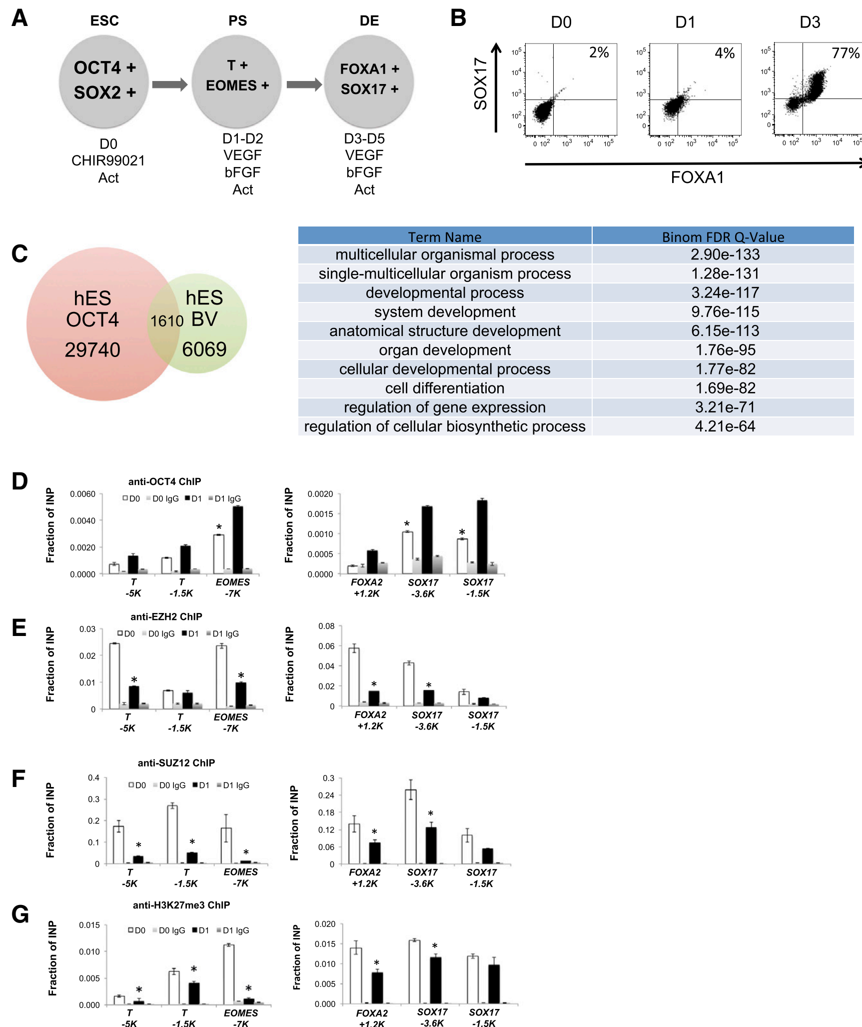
A growing body of evidence underscores the importance of pluripotency factors during differentiation. Human and mouse ES cell studies have demonstrated that the core pluripotency transcription factors, SOX2, OCT4, and NANOG, play distinct roles in coordinating ES cell lineage commitment (Lu et al., 2009; Thomson et al., 2011; Wang et al., 2012). *NANOG* promotes definitive endoderm (DE) formation by coordinating with the activation of the TGF-β signaling pathway through the induction of *EOMES* (Teo et al., 2011). *SOX2* is important in ectoderm formation by limiting mesendoderm formation (Wang et al., 2012).

The role of OCT4 in lineage commitment is somewhat less clear. In zebrafish, the *OCT4* homolog *spg* is required for endoderm induction (Lunde et al., 2004). The role of OCT4 during human ES (hES) cell differentiation and lineage commitment is controversial because knockdown studies performed by different labs have resulted in the induction of trophectoderm, primitive endoderm, or neuroectoderm cell fates (Niwa et al., 2000; Wang et al., 2012). Another study suggested that *OCT4* knockdown induced DE formation, even though gene expression was low (Teo et al., 2011). These studies also demonstrated a critical role for OCT4 in maintaining the undifferentiated state.

Several studies have demonstrated that OCT4 may play a role in regulating the epigenetic landscape in ES cells and during differentiation. Pull-down assays indicate that major Oct4 partners in mouse ES cells are related to chromatin remodeling (Pardo et al., 2010; van den Berg et al., 2010). Bernstein et al. (2006) defined a specific chromatin modification pattern called the "bivalent domain," which harbors both the inhibitory, H3K27me3, and activating, H3K4me3, histone modifications at genes important in regulating early development in ES cells. Genes present in bivalent domains are typically silent in ES cells, but are poised for activation. Genome-wide studies have shown that OCT4 co-localizes with Polycomb2 (PRC2) components, which are responsible for laying down the inhibitory H3K27me3 mark and generating bivalent domains in hES cells (Boyer et al., 2005, 2006). Based on these findings, OCT4 may play an important role in chromatin remodeling during differentiation in response to external signals.

The Wnt signaling pathway is important for both maintaining pluripotency (Sokol, 2011; Wang and Wynshaw-Boris, 2004) and inducing differentiation to primitive streak and mesendoderm (Cheng et al., 2008; Gadue et al., 2006; Lyashenko et al., 2011). In mouse ES cells, Oct4 has been shown to play a role in the Wnt signaling pathway by physically interacting with β-catenin to reinforce pluripotency (Kelly et al., 2011).

CrossMark



Term Name	Binom FDR Q-Value
multicellular organismal process	2.90e-133
single-multicellular organism process	1.28e-131
developmental process	3.24e-117
system development	9.76e-115
anatomical structure development	6.15e-113
organ development	1.76e-95
cellular developmental process	1.77e-82
cell differentiation	1.69e-82
regulation of gene expression	3.21e-71
regulation of cellular biosynthetic process	4.21e-64

Figure 1. Efficient Differentiation of hES Cells into DE

(A) Scheme of endodermal differentiation.
(B) Flow cytometry analysis of hES cells and D0, D1, and D3 of endoderm differentiation cultures with anti-SOX17 and anti-FOXA1 antibodies.
(C) (Left) Venn diagram of OCT4 and bivalent domain occupancy in hES cells. (Right) GO terms from the gene set overlapping OCT4 and bivalent domains.
(D) Anti-OCT4.
(E) Anti-EZH2.
(F) Anti-SUZ12.
(G) Anti-H3K27me3 ChIP on H9 D0 and D1 cells. Sites used for ChIP-qPCR are indicated in kilobases (K) from the transcription start site. The IgG control for each ChIP is also shown. Data shown are an average from three independent experiments. Statistical significance indicated as *$p < 0.05$. Activin A, Act; input, INP.

In this study, we define a role for OCT4 and Wnt signaling in establishing an appropriate chromatin signature during hES cell specification into endoderm. By utilizing a directed differentiation approach coupled with siRNA knockdown of *OCT4*, we show that OCT4 and Wnt signaling play a role in the chromatin pre-patterning of endoderm genes such as *SOX17* and *FOXA2*. During mesendoderm commitment, OCT4 physically associates with β-catenin while the knockdown of *OCT4* eliminates the mesendoderm differentiation capacity of hES cells.

OCT4 knockdown also led to a failure to remove the PRC2 complex from primitive streak and endodermal genes. In the absence of Wnt pathway activation during endoderm induction, hES cells maintain high levels of OCT4 protein but fail to evict the PRC2 complex and downregulate H3K27me3 on primitive streak and endodermal genes. In summary, OCT4 plays a key role in the pluripotency core network and is indispensable for lineage commitment by coordinating with WNT signaling to target bivalent genes for activation. These data underscore the importance of pluripotent transcription factors in differentiation as well as for maintenance of the pluripotent state.

RESULTS

Dynamic Changes of Pluripotency and Primitive Streak Genes during DE Differentiation of hES Cells

H9 hES cells were differentiated toward DE using a previously described protocol (Nostro et al., 2008) that utilizes high levels of activin A and Wnt pathway activation with the small molecule CHIR99021 (CHIR) (Figure 1A). Confirming previous findings (Teo et al., 2011), we see that the level of two pluripotency genes, *OCT4* and *NANOG*, is transiently increased at day 1 of differentiation before subsequently declining (Figures S1A and S1B). In contrast, *SOX2* is progressively lost through all days of the differentiation protocol (Figures S1A and S1B). Primitive streak

genes such as *T*, *GSC*, and *EOMES* were quickly induced by D1 of differentiation (Figure S1B). By days 2 and 3 of differentiation, DE genes, such as *FOXA1*, *FOXA2*, and *SOX17* were expressed which represents commitment to DE (Figures 1B and S1B).

To study the mechanism of OCT4 involvement during endoderm induction, we took advantage of existing genome-wide ChIP-seq (Guenther et al., 2010; Zhao et al., 2007) and transcription profile datasets (Cheng et al., 2012) to analyze the co-localization of OCT4 binding and histone modification sites. The analysis of previously published data revealed that >25% of defined “bivalent domains” co-localize with OCT4 binding sites (Zhao et al., 2007) (Figure 1C). Gene Ontology (GO) analysis of these sites showed an enrichment of terms related to development and differentiation (Figure 1C). We hypothesized that OCT4 may play a role in the regulation of these bivalent domains to affect early differentiation. ChIP analyses were performed on ES cell cultures at D0 and D1 of differentiation, a time point when early primitive streak markers are induced but the majority of endodermal genes are not yet expressed. The ChIP primers were designed to detect OCT4 binding sites within bivalent domains of primitive streak (*T* and *EOMES*) and DE genes (*FOXA2* and *SOX17*). OCT4 binding increased on both primitive streak and endoderm gene sites at D1 of differentiation (Figure 1D). We examined occupancy of EZH2 and SUZ12, two components of the PRC2 complex, and the H3K27me3 inhibitory histone mark at these same OCT4 binding sites. On the primitive streak gene loci, the binding of EZH2, SUZ12, and the H3K27me3 mark decreased at day 1 of differentiation (Figures 1E–1G), correlating well with the induction of these genes as shown in Figure S1B. We found similar decreases in EZH2 and SUZ12 occupancy and the H3K27me3 mark on DE genes at D1 of differentiation, prior to *SOX17* gene expression (Figures 1E–1G). We also examined a second WT iPS cell line, CHOPWT2.1, for the H3K27me3 mark and see similar results (Figure S1C). The expression of *EZH2* and *SUZ12* does not significantly decline at D1 of differentiation at the mRNA or protein level (Figure S1E), and SUZ12 and H3K27me3 were not depleted on neuroectodermal genes at D1, such as *PAX6* and *OLIGO1* (Figure S1D), suggesting that the PRC2 complex is not globally decreased at this time point. These data support the idea that OCT4 may play a role in evicting the PRC2 complex specifically from mesendodermal and definitive endodermal genes, priming them for expression.

OCT4 Interacts with β-Catenin to Pre-pattern the Epigenetic Profile of hES Cells during DE Differentiation

Wnt signaling has a well-established role in primitive streak and mesendoderm formation in the mammalian embryo and in ES cell differentiation cultures (Gadue et al., 2005). Therefore, we examined this signaling pathway and its interactions with OCT4 during endoderm induction in the hES cell differentiation system. Activated β-catenin (ABC) increased in response to Wnt signaling induced by the GSK3 inhibitor, CHIR, during ES cell differentiation (Kunisada et al., 2012) (Figure S1A), and β-catenin was shown to translocate from the cytoplasm to the nucleus (Figure 2A).

To determine whether β-catenin activity is important for epigenetic changes during differentiation, we performed ChIP assays on hES cells at D1 of differentiation in the absence or presence of CHIR. Without CHIR, OCT4 recruitment to primitive streak and DE genes (Figure 2B, i and ii) is impaired. The decreased OCT4 binding on primitive streak and DE genes correlates with increased occupancy of EZH2 (Figure 2B, iii and iv), SUZ12 (Figure 2B, v and vi) and H3K27me3 (Figure 2B, vii and viii) on these sites in the absence of CHIR without a global change of *EZH2* or *SUZ12* levels (Figure S1F, right). This increased occupancy is also seen on another primitive streak gene, *GSC* (Figure S1F).

While it is well established that transient Wnt induction is important for mesendoderm differentiation from hES cells, we examined the impact on OCT4 expression in the initial 24 hr of differentiation. In the absence of CHIR, we still find upregulation of OCT4 at the protein (Figure 2C) and RNA (Figure 2D) levels. In contrast, SOX2 is not downregulated, and primitive streak genes are poorly upregulated in the absence of CHIR (Figures 2C and 2D). While not detectable at the protein level at D1 of differentiation (Figure 1B), *FOXA1* and *FOXA2* mRNAs were also not upregulated in the absence of Wnt activation by CHIR (Figure 2D). Similar results were obtained with an additional human iPS cell line (Figures S1G and S1H). These data suggest that Wnt signaling is critical for mesendoderm gene induction but does not impact *OCT4* expression at this early stage of differentiation.

We hypothesized that Wnt signaling components may be interacting with OCT4 to promote mesendoderm induction. Using nuclear extracts, we found an enhanced physical interaction between OCT4 and β-catenin by co-immunoprecipitation analysis at D1 of differentiation using antibodies for either total or activated β-catenin (Figure 2E). These data suggest that β-catenin and OCT4 may act in concert to regulate the removal of the PRC2 complex on primitive streak and endoderm genes, priming them for expression.

OCT4 Is Indispensable for Primitive Streak Commitment and DE Differentiation

To examine the role of OCT4 during endoderm differentiation, we transiently knocked down *OCT4* with siRNA in hES cells or human iPS cells prior to the start of

Figure 2. WNT Pathway Activation Is Required for Primitive Streak Commitment and Endoderm Differentiation

(A) Immunofluorescence staining of anti-OCT4 (green) and anti-ABC (red) on D0 and D1 differentiation cultures performed as described in Figure 1, counterstained with DAPI (blue) for nuclei. Distribution of fluorescence intensity across a single representative cells was measured and plotted, demonstrating nuclear localization of ABC at D1. Scale bar represents for 100 μm.

(B) ChIP-qPCR analysis comparing D1 differentiation cultures with and without the Wnt agonist CHIR99021 (Chir) on primitive streak genes (*T* and *EOMES*) and endodermal genes (*FOXA2* and *SOX17*). (i) and (ii) anti-OCT4, (iii) and (iv) anti-EZH2, (v) and (vi) anti-SUZ12,

(legend continued on next page)

differentiation. An *OCT4* knockdown time course showed that though *OCT4* mRNA level decreased 4 hr after transfection (Figure S2A, i), 18 hr was required for maximal effect at the protein level (Figure S2, ii). Therefore, *OCT4* knockdown was performed 18 hr prior to differentiation in subsequent experiments. While the siRNA treatment led to a dramatic decrease in OCT4 protein (Figures 3A and S3A, i) and RNA levels (Figure S2A, i), other pluripotent markers such as NANOG and SOX2 displayed only mildly decreased expression at the protein level in the H9 hES cell line (Figure S2A, iii and iv) and remained unchanged in the human iPS cells line, CHOPWT2.1 (Figure S3A, ii and iii). OCT4 knockdown cells also retained other ES cell markers such as Tra-1-81 and SSEA3 (Figure S2A, v). Consistent with a previous study (Teo et al., 2011), the expression of primitive streak and DE markers were also detected 72 hr after *OCT4* siRNA transfection in ES cell culture media, but the levels of expression for these genes were extremely low compared with the expression levels of these genes during the differentiation to mesendoderm in D1 cells (Figure S2B, ii) and DE in D3 cells (Figure S2B, iii). These data suggest that the loss of *OCT4* without differentiation induction may cause transcriptional de-repression of lineage-specific genes as opposed to rapid activation to physiologically relevant levels. When *OCT4* siRNA-transfected hESCs were subjected to DE differentiation, primitive streak genes, such as *T*, *EOMES*, and *GSC*, were not fully activated (Figures 3A, 3B, and S2C). DE genes, such as *CXCR4*, *CD117*, *FOXA1*, *FOXA2*, and *SOX17*, were also poorly induced (Figures 3C, S2A, vi, S2C, and S3B, i, ii, and iii). These findings were confirmed using a second *OCT4* siRNA (Figure S2D). These data indicate that *OCT4* expression is necessary for primitive streak and endoderm induction during hES cell and iPS cell differentiation.

OCT4 Regulates PRC2 Complex Recruitment during Mesendoderm Commitment

To determine whether OCT4 was necessary for the change in PRC2 complex occupancy of endodermal genes following differentiation, the H9 hES cell line or human iPS cell line CHOPWT2.1 was treated with *OCT4* siRNA and examined at D1 of endoderm differentiation by ChIP. In the absence of OCT4, the occupancy of EZH2 and SUZ12 on primitive streak genes was increased (Figures 3D, 3E, S2E, and S3C, i) without global changes in EZH2 or SUZ12 protein levels (Figure S2F). Importantly, EZH2 and SUZ12 were significantly increased on at least one site on the *FOXA2* and *SOX17* loci (Figures 3D and 3E, and S3Ci), which correlated with higher levels of the H3K27me3 mark (Figures 3F and S3C, ii) and loss of *FOXA1* and *SOX17* gene expression at D3 of differentiation (Figures 3B, 3C, and S3B, ii and iii). These findings suggest that OCT4 is critical for PRC2 complex-mediated epigenetic pre-patterning of the master endoderm regulator *SOX17* prior to gene expression.

DISCUSSION

The pluripotency status of ES cells or iPS cells is maintained by a tightly regulated transcription factor network (Boyer et al., 2005). OCT4 is at the center of this network (Pardo et al., 2010; van den Berg et al., 2010) and is associated with chromatin modifiers to maintain epigenetic identity of pluripotent cells (Liang et al., 2008; Zhao et al., 2007). Its expression level is strictly controlled in pluripotent cells in which changes in protein level, either higher or lower, can lead to lineage-specific differentiation (Niwa et al., 2000; Wang et al., 2012).

Wnt signaling is well known as an inducer of primitive streak formation (Gadue et al., 2006) and an indispensable component for efficient endoderm differentiation (Han et al., 2011). Endogenous WNT3 expression in ES cells can predict endoderm differentiation efficiency, highlighting the importance of this pathway in endoderm induction (Jiang et al., 2013). While Wnt can directly activate primitive streak genes such as *T* (Gadue et al., 2006), we demonstrate a secondary effect that this pathway has on regulating OCT4 function. Activation of β-catenin was critical for increased OCT4 binding to target genes and presumably regulates the transcription status of these genes during activin A-induced differentiation. Without Wnt signaling, mesendoderm genes (*T*, *EOMES*, and *GSC*) and DE genes (*FOXA2* and *SOX17*) maintain the repressive PRC2 complex and the H3K27me3 mark. These findings are supported by recent work in the mouse ES cell system where it was demonstrated that a composite *Oct4-Tcf/Lef* site was critical for Mesp1 induction and cardiomyocyte

(vii) and (viii) anti-H3K27me3. Sites used for ChIP-qPCR are indicated in kilobases (K) from the transcription start site. Data shown are an average from three independent experiments. Statistical significance represented as *$p < 0.05$.
(C) Flow cytometry analysis on D0 and D1 differentiation cultures with or without Chir addition, examining SOX2, OCT4, EOMES, and T expression.
(D) QRT-PCR analysis of D1 differentiation cultures with or without Chir addition.
(E) Immunoprecipitation of nuclear extracts from hES cells and D1 differentiation cultures with anti-OCT4 antibody and blotted with (left) anti-β-CATENIN and anti-OCT4 antibodies or (right) anti-active β-Catenin and anti-OCT4 antibodies; 1% of total lysate was run in the input lanes. Immunoprecipitation, IP; skipped lane, −; input, INP.

Figure 3. OCT4 Knockdown Eliminates Endoderm Differentiation

HES cells were transfected with siRNA against *OCT4* or a control scramble siRNA construct 18 hr prior to initiation of endoderm differentiation as described in Figure 1.

(A) Flow cytometry analysis of OCT4 protein levels at D0, and T and EOMES levels at D1 of endoderm differentiation.

(B) QRT-PCR gene expression analysis of *T* and *EOMES* at D1 and *FOXA1* and *SOX17* at D3 of differentiation. Data shown are an average three independent experiments. Statistical significance represented as $*p < 0.05$.

(C) Flow cytometry analysis of CXCR4, FOXA1, and SOX17 at D3 of endoderm differentiation.

(legend continued on next page)

differentiation (Li et al., 2013). Based on our observations, we propose a preliminary working model of how OCT4 cooperates with β-catenin to evict the PRC2 complex and remove the repressive histone mark at differentiation initiation (Figure S3D).

Two recent studies have reported opposite conclusions on the effect of *OCT4* knockdown in hES cell differentiation. Teo et al. (2011) demonstrated that *OCT4* knockdown led to precocious upregulation of endodermal genes, while Wang et al. (2012) reported that *OCT4* knockdown inhibits mesendoderm differentiation. Our data support the findings from both sides and shed light on the mechanism of these seemingly contradictory results. Like Teo et al., we also find endodermal gene expression upon *OCT4* knockdown. However, the level of endodermal and primitive streak gene induction, while increased over that seen in ES cells, was orders of magnitude less than expression of these genes during normal differentiation (Figure S2B, ii, iii). In both hES cells and iPS cells, when *OCT4* knockdown cells were subjected to DE differentiation, expression of primitive streak and DE genes was drastically downregulated compared with scramble siRNA transfected cells, which supported the findings of Wang et al. (2012). These seemingly contradictory phenotypes of *OCT4* knockdown may underlie multiple roles for OCT4 in ES cell maintenance and differentiation. OCT4 is well known to repress differentiation genes (Boyer et al., 2005) such that lack of its expression leads to de-repression of these genes but at low levels without other differentiation induction signals. The second role of OCT4 that we uncovered is its involvement in the removal of PRC2 complex from mesendodermal genes and its requirement for the physiologic expression of these genes during directed differentiation of hES cells.

Recent studies examining the role of *Oct4* showed that *Oct4* is critical in lineage specification in the mouse, especially for the endodermal program and supports our results. $Oct4^{+/-}$ ES cells and mouse iPS cells expressing low levels of *Oct4* can be maintained in the undifferentiated state and express increased levels of many pluripotency markers (Karwacki-Neisius et al., 2013; Radzisheuskaya et al., 2013). Both pluripotent stem cell lines also displayed an inhibition of differentiation into embryonic lineages. In addition, mouse blastocysts that lack Oct4 fail to upregulate primitive endoderm genes in a cell autonomous manner. Our results show that OCT4 is not only critical for mesendoderm gene activation, but also crucial for DE gene expression by pre-patterning epigenetic marks prior to gene expression.

In summary, we demonstrated that OCT4 is critical for mesendoderm differentiation by showing its involvement in eviction of the PRC2 complex and loss of the H3K27me3 mark from mesendodermal genes. The effect on *SOX17* is most revealing as it demonstrates pre-patterning of this gene prior to gene expression that may be critical for eventual endoderm germ layer formation. This effect requires Wnt signaling, giving a mechanism for how an external stimulus can allow a pluripotency factor, OCT4, to behave as a differentiation factor. This study highlights the multiple roles that pluripotency genes play in maintaining the ES cell state but also in directing subsequent differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation

The H9 hES cell line was obtained from the Wicell Research Institute. The CHOPWT2.1 iPS cell line was described previously (Mills et al., 2013). hES and iPS cell culture and differentiation followed previous paper (Cheng et al., 2012; Nostro et al., 2008). hES cells and human iPS cells were cultured in DMEM/F12 supplemented with 15% knockout serum replacement (KSR) and 10 ng/ml basic fibroblast growth factor (bFGF) on mouse embryonic feeders (MEFs). hES cells and iPS cells were replated onto matrigel-coated surface to perform monolayer differentiation. Briefly, the GSK3 inhibitor CHIR99021 and activin A were added at day 0 (D0) to induce differentiation initiation. Then medium containing 0.25 ng/ml BMP4, 5 ng/ml bFGF, 10 ng/ml vascular endothelial growth factor (VEGF), and 100 ng/ml activin A was added to induce DE.

siRNA Knockdown

OCT4 and control scramble siRNAs were obtained from Integrated DNA Technologies. Sequences of siRNAs are listed in Table S1. H9 hES cells were replated onto matrigel-coated plate 24 hr before transfection. When H9 hES cell culture reached 70% confluent, siRNA transfection was carried out with Lipofectamine RNAiMAX (Invitrogen). Transfected cells were either cultured in hES media or DE differentiation culture conditions 18 hr after transfection.

ChIP-qPCR, Flow Cytometry, Immunofluorescence, Statistics, Western Blot, and Immunoprecipitation

Detailed procedures are listed in Supplement Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.08.014.

(D–F) ChIP-qPCR analysis comparing D1 differentiation cultures treated with *OCT4* or scramble siRNA on primitive streak genes (*T* and *EOMES*) and endodermal genes (*FOXA2* and *SOX17*). (D) Anti-EZH2. (E) Anti-SUZ12. (F) Anti-H3K27me3. Sites used for ChIP-qPCR are indicated in kilobases (K) from the transcription start site. Data shown are an average from three independent experiments. Statistical significance represented as $^*p < 0.05$.

AUTHOR CONTRIBUTIONS

L.Y. collected and/or assembled data, performed data analysis and interpretation, and wrote the manuscript. J.A.M. collected and/or assembled data and performed data analysis and interpretation. D.L.F. performed data analysis and interpretation and wrote the manuscript. P.G. performed data analysis and interpretation, wrote the manuscript, and gave final approval of the manuscript.

ACKNOWLEDGMENTS

This work was supported by NIH grant R01 DK092113.

Received: August 29, 2014
Revised: August 25, 2015
Accepted: August 26, 2015
Published: September 24, 2015

REFERENCES

Bernstein, B.E., Mikkelsen, T.S., Xie, X., Kamal, M., Huebert, D.J., Cuff, J., Fry, B., Meissner, A., Wernig, M., Plath, K., et al. (2006). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell *125*, 315–326.

Boyer, L.A., Lee, T.I., Cole, M.F., Johnstone, S.E., Levine, S.S., Zucker, J.P., Guenther, M.G., Kumar, R.M., Murray, H.L., Jenner, R.G., et al. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. Cell *122*, 947–956.

Boyer, L.A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L.A., Lee, T.I., Levine, S.S., Wernig, M., Tajonar, A., Ray, M.K., et al. (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature *441*, 349–353.

Cheng, X., Huber, T.L., Chen, V.C., Gadue, P., and Keller, G.M. (2008). Numb mediates the interaction between Wnt and Notch to modulate primitive erythropoietic specification from the hemangioblast. Development *135*, 3447–3458.

Cheng, X., Ying, L., Lu, L., Galvão, A.M., Mills, J.A., Lin, H.C., Kotton, D.N., Shen, S.S., Nostro, M.C., Choi, J.K., et al. (2012). Self-renewing endodermal progenitor lines generated from human pluripotent stem cells. Cell Stem Cell *10*, 371–384.

Gadue, P., Huber, T.L., Nostro, M.C., Kattman, S., and Keller, G.M. (2005). Germ layer induction from embryonic stem cells. Exp. Hematol. *33*, 955–964.

Gadue, P., Huber, T.L., Paddison, P.J., and Keller, G.M. (2006). Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. Proc. Natl. Acad. Sci. USA *103*, 16806–16811.

Guenther, M.G., Frampton, G.M., Soldner, F., Hockemeyer, D., Mitalipova, M., Jaenisch, R., and Young, R.A. (2010). Chromatin structure and gene expression programs of human embryonic and induced pluripotent stem cells. Cell Stem Cell *7*, 249–257.

Han, S., Dziedzic, N., Gadue, P., Keller, G.M., and Gouon-Evans, V. (2011). An endothelial cell niche induces hepatic specification through dual repression of Wnt and Notch signaling. Stem Cells *29*, 217–228.

Jiang, W., Zhang, D., Bursac, N., and Zhang, Y. (2013). WNT3 is a biomarker capable of predicting the definitive endoderm differentiation potential of hESCs. Stem Cell Reports *1*, 46–52.

Karwacki-Neisius, V., Göke, J., Osorno, R., Halbritter, F., Ng, J.H., Weiße, A.Y., Wong, F.C., Gagliardi, A., Mullin, N.P., Festuccia, N., et al. (2013). Reduced Oct4 expression directs a robust pluripotent state with distinct signaling activity and increased enhancer occupancy by Oct4 and Nanog. Cell Stem Cell *12*, 531–545.

Kelly, K.F., Ng, D.Y., Jayakumaran, G., Wood, G.A., Koide, H., and Doble, B.W. (2011). β-catenin enhances Oct-4 activity and reinforces pluripotency through a TCF-independent mechanism. Cell Stem Cell *8*, 214–227.

Kunisada, Y., Tsubooka-Yamazoe, N., Shoji, M., and Hosoya, M. (2012). Small molecules induce efficient differentiation into insulin-producing cells from human induced pluripotent stem cells. Stem Cell Res. (Amst.) *8*, 274–284.

Li, Y., Yu, W., Cooney, A.J., Schwartz, R.J., and Liu, Y. (2013). Brief report: Oct4 and canonical Wnt signaling regulate the cardiac lineage factor Mesp1 through a Tcf/Lef-Oct4 composite element. Stem Cells *31*, 1213–1217.

Liang, J., Wan, M., Zhang, Y., Gu, P., Xin, H., Jung, S.Y., Qin, J., Wong, J., Cooney, A.J., Liu, D., and Songyang, Z. (2008). Nanog and Oct4 associate with unique transcriptional repression complexes in embryonic stem cells. Nat. Cell Biol. *10*, 731–739.

Lu, R., Markowetz, F., Unwin, R.D., Leek, J.T., Airoldi, E.M., MacArthur, B.D., Lachmann, A., Rozov, R., Ma'ayan, A., Boyer, L.A., et al. (2009). Systems-level dynamic analyses of fate change in murine embryonic stem cells. Nature *462*, 358–362.

Lunde, K., Belting, H.G., and Driever, W. (2004). Zebrafish pou5f1/pou2, homolog of mammalian Oct4, functions in the endoderm specification cascade. Curr. Biol. *14*, 48–55.

Lyashenko, N., Winter, M., Migliorini, D., Biechele, T., Moon, R.T., and Hartmann, C. (2011). Differential requirement for the dual functions of β-catenin in embryonic stem cell self-renewal and germ layer formation. Nat. Cell Biol. *13*, 753–761.

Mills, J.A., Wang, K., Paluru, P., Ying, L., Lu, L., Galvão, A.M., Xu, D., Yao, Y., Sullivan, S.K., Sullivan, L.M., et al. (2013). Clonal genetic and hematopoietic heterogeneity among human-induced pluripotent stem cell lines. Blood *122*, 2047–2051.

Murry, C.E., and Keller, G. (2008). Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell *132*, 661–680.

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.

Nostro, M.C., Cheng, X., Keller, G.M., and Gadue, P. (2008). Wnt, activin, and BMP signaling regulate distinct stages in the developmental pathway from embryonic stem cells to blood. Cell Stem Cell *2*, 60–71.

Pardo, M., Lang, B., Yu, L., Prosser, H., Bradley, A., Babu, M.M., and Choudhary, J. (2010). An expanded Oct4 interaction network: implications for stem cell biology, development, and disease. Cell Stem Cell *6*, 382–395.

Radzisheuskaya, A., Chia, Gle.B., dos Santos, R.L., Theunissen, T.W., Castro, L.F., Nichols, J., and Silva, J.C. (2013). A defined

Oct4 level governs cell state transitions of pluripotency entry and differentiation into all embryonic lineages. Nat. Cell Biol. *15*, 579–590.

Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A., and Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat. Biotechnol. *18*, 399–404.

Sokol, S.Y. (2011). Maintaining embryonic stem cell pluripotency with Wnt signaling. Development *138*, 4341–4350.

Teo, A.K., Arnold, S.J., Trotter, M.W., Brown, S., Ang, L.T., Chng, Z., Robertson, E.J., Dunn, N.R., and Vallier, L. (2011). Pluripotency factors regulate definitive endoderm specification through eomesodermin. Genes Dev. *25*, 238–250.

Thomson, M., Liu, S.J., Zou, L.N., Smith, Z., Meissner, A., and Ramanathan, S. (2011). Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. Cell *145*, 875–889.

van den Berg, D.L., Snoek, T., Mullin, N.P., Yates, A., Bezstarosti, K., Demmers, J., Chambers, I., and Poot, R.A. (2010). An Oct4-centered protein interaction network in embryonic stem cells. Cell Stem Cell *6*, 369–381.

Wang, J., and Wynshaw-Boris, A. (2004). The canonical Wnt pathway in early mammalian embryogenesis and stem cell maintenance/differentiation. Curr. Opin. Genet. Dev. *14*, 533–539.

Wang, Z., Oron, E., Nelson, B., Razis, S., and Ivanova, N. (2012). Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells. Cell Stem Cell *10*, 440–454.

Zhao, X.D., Han, X., Chew, J.L., Liu, J., Chiu, K.P., Choo, A., Orlov, Y.L., Sung, W.K., Shahab, A., Kuznetsov, V.A., et al. (2007). Whole-genome mapping of histone H3 Lys4 and 27 trimethylations reveals distinct genomic compartments in human embryonic stem cells. Cell Stem Cell *1*, 286–298.

Stem Cell Reports, Volume *5*

Supplemental Information

OCT4 Coordinates with WNT Signaling to Pre-pattern Chromatin at the *SOX17* Locus during Human ES Cell Differentiation into Definitive Endoderm

Lei Ying, Jason A. Mills, Deborah L. French, and Paul Gadue

Supplemental Figures

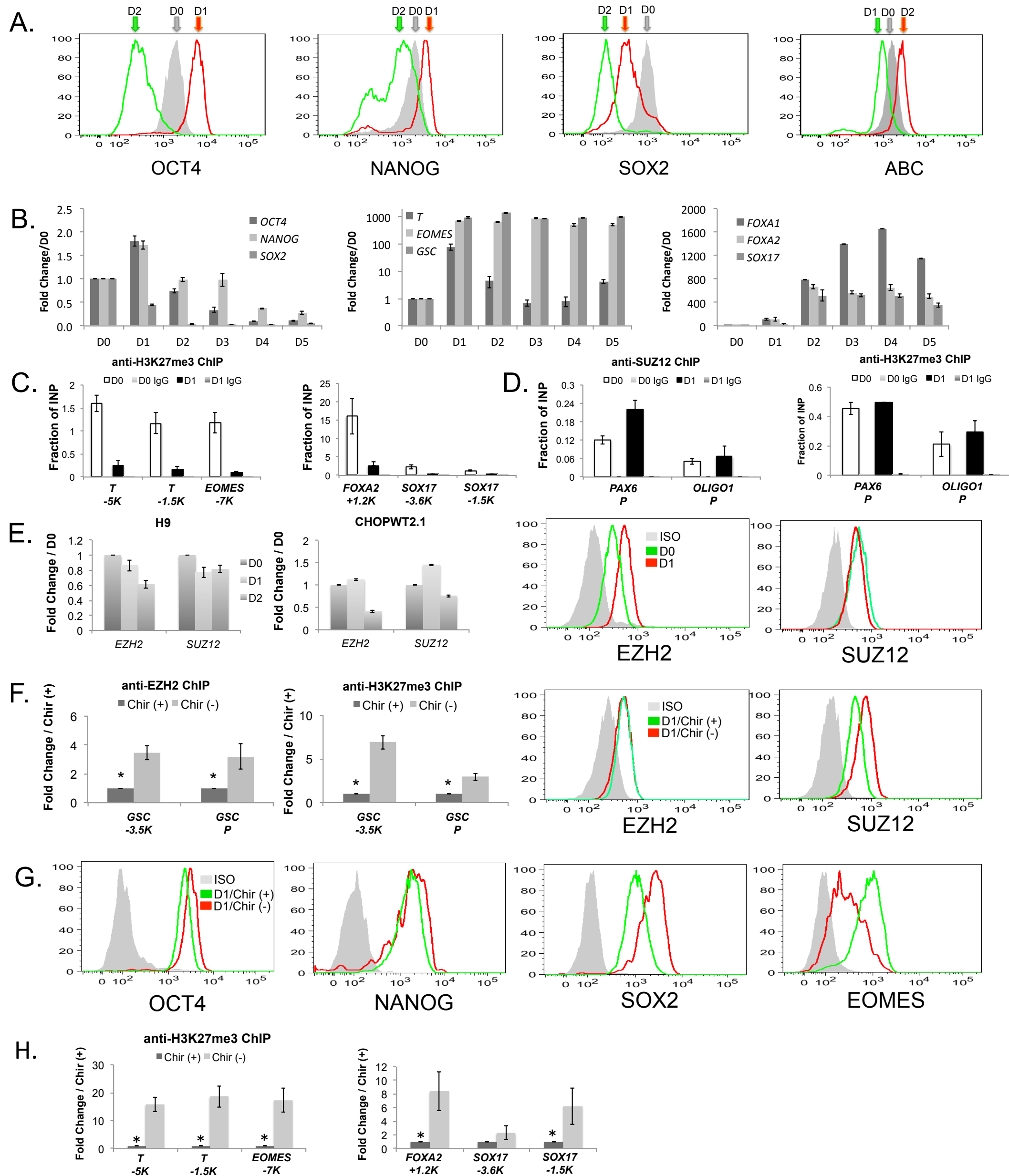
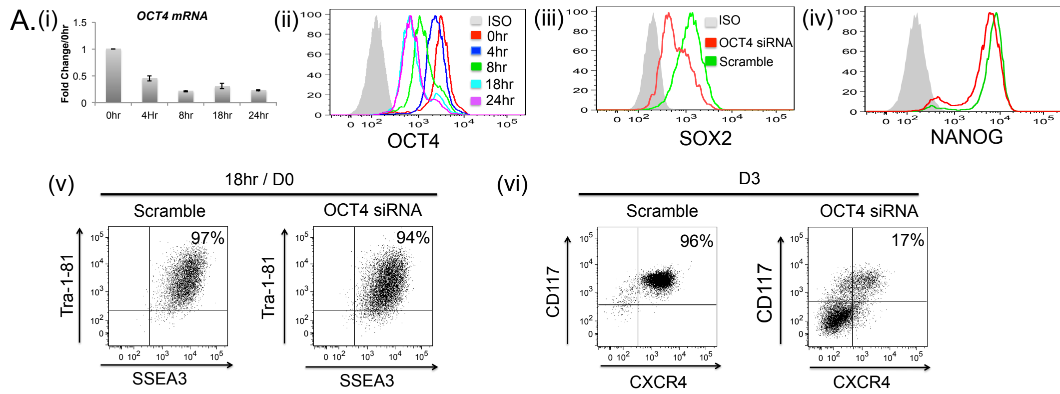
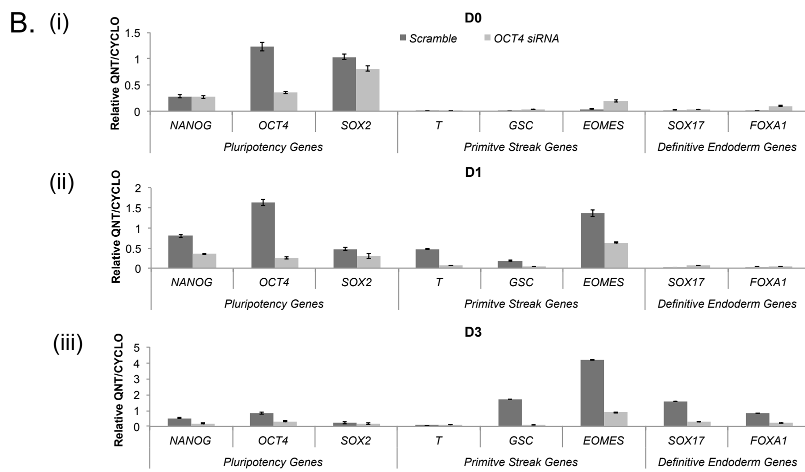


Figure S1: Gene expression profiles and ChIP-qPCR data during endoderm differentiation.

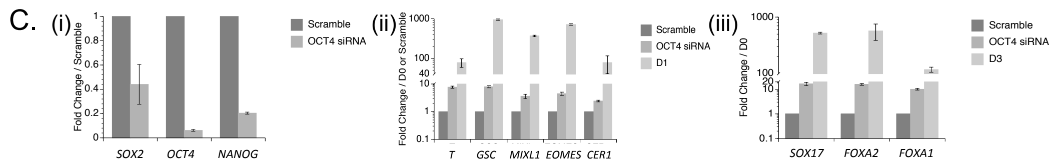
A. Flow cytometry analysis of OCT4, NANOG and SOX2 and activated β-Catenin (ABC) on D0, D1 and D2 H9 endoderm differentiation cultures. B. Time course qRT-PCR expression profiles of pluripotency genes (*OCT4, NANOG* and *SOX2*), primitive streak genes (*T, EOMES,* and *GSC*) and endodermal genes (*FOXA1, FOXA2* and *SOX17*) in H9 endoderm differentiation cultures. C. Anti-H3K27me3 ChIP-qPCR analysis on regulatory regions of primitive streak genes (*T* and *EOMES*) and endodermal genes (*FOXA2* and *SOX17*) in CHOPWT2.1 iPS cell endoderm differentiation cultures on D0 and D1. D. Anti-SUZ12 ChIP-qPCR on neuroectoderm gene loci in H9 hES cell differentiation cultures at D0 and D1. E. (left panel) *EZH2* and *SUZ12* qRT-PCR expression profiles in H9 and CHOPWT2.1 differentiation cultures on D0, D1 and D2. (right panel) EZH2 and SUZ12 intracellular flow cytometric analysis on H9 hES cells at D0 and D1. F. (left panel) Anti-EZH2 and anti-H3K27me3 ChIP-qPCR on D1 endoderm differentiation cultures of H9 ES cells treated with and without Chir. (right panel) EZH2 and SUZ12 intracellular flow cytometry on D1 endoderm differentiation cultures of H9 ES cells treated with and without Chir. Data shown are from three independent experiments. Statistical significance represented as * for $p < 0.05$.G. Flow cytometry analysis of OCT4, NANOG, SOX2 and EOMES on D1 of CHOPWT2.1 iPS cell differentiation cultures with and without Chir. H. Anti-H3K27me3 ChIP-qPCR in CHOPWT2.1 iPS cell differentiation cultures on D1 with and without Chir. Sites used for ChIP-qPCR are indicated in kilobases (K) from the transcription start site. Data shown are from three independent experiments. Statistical significance indicated as * for $p < 0.05$.



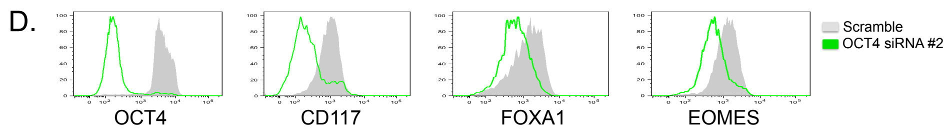
A. (i)
OCT4 mRNA
Fold Change/0hr
0hr
4Hr
8hr
18hr
24hr
(ii)
ISO
0hr
4hr
8hr
18hr
24hr
OCT4
(iii)
ISO
OCT4 siRNA
Scramble
SOX2
(iv)
NANOG
(v)
18hr / D0
Scramble
OCT4 siRNA
97%
94%
Tra-1-81
SSEA3
(vi)
D3
Scramble
OCT4 siRNA
96%
17%
CD117
CXCR4



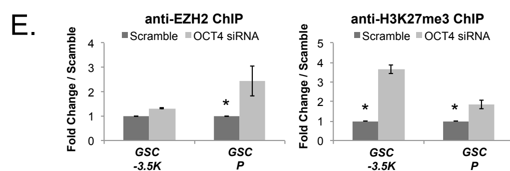
B. (i)
D0
Scramble
OCT4 siRNA
Relative QNT/CYCLO
NANOG
OCT4
SOX2
T
GSC
EOMES
SOX17
FOXA1
Pluripotency Genes
Primitve Streak Genes
Definitive Endoderm Genes
(ii)
D1
(iii)
D3



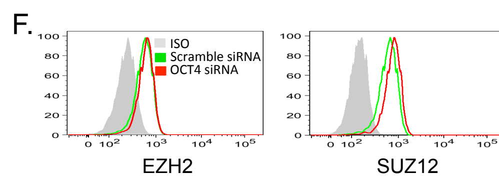
C. (i)
Fold Change / Scramble
Scramble
OCT4 siRNA
SOX2
OCT4
NANOG
(ii)
Fold Change / D0 or Scramble
Scramble
OCT4 siRNA
D1
T
GSC
MIXL1
EOMES
CER1
(iii)
Fold Change / D0
Scramble
OCT4 siRNA
D3
SOX17
FOXA2
FOXA1



D.
OCT4
CD117
FOXA1
EOMES
Scramble
OCT4 siRNA #2

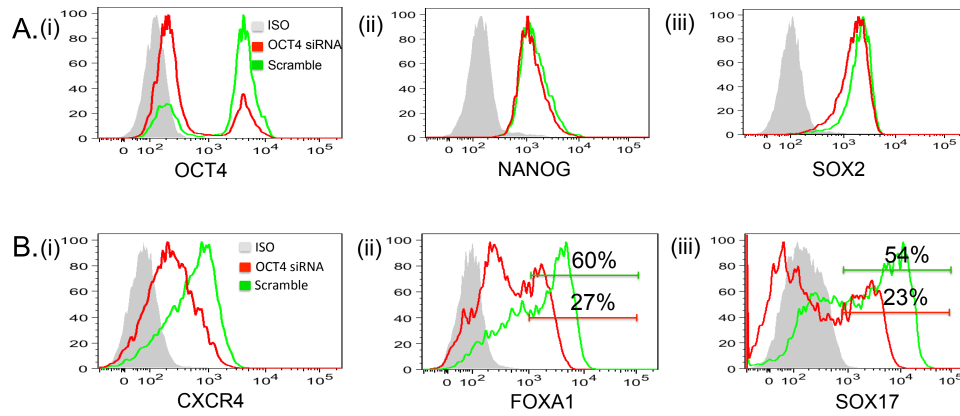


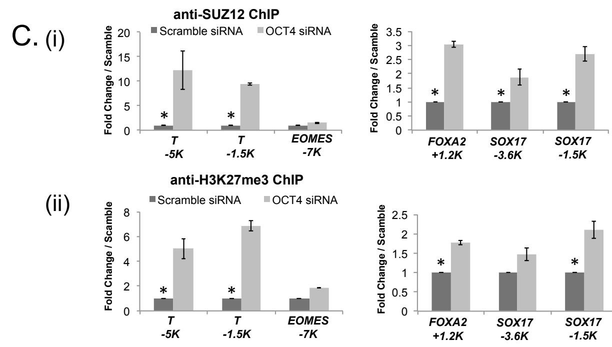
E.
anti-EZH2 ChIP
Scramble
OCT4 siRNA
Fold Change / Scramble
GSC -3.5K
GSC P
anti-H3K27me3 ChIP
Fold Change / Scamble



F.
ISO
Scramble siRNA
OCT4 siRNA
EZH2
SUZ12

Figure S2. Gene expression profile and ChIP-qPCR after *OCT4* knockdown. A. (i) QRT-PCR and (ii) Flow cytometry time course of OCT4 siRNA knockdown in H9 ES cells. (iii-vi) Flow cytometry analysis of pluripotency markers (iii) SOX2, (iv) NANOG, (v) SSEA3 and TRA-1-81 on H9 hES cells transfected with scramble or *OCT4*-siRNA at D0 (18 hours after transfection) and (vi) definitive endoderm markers, CXCR4 and CD117, on D3 endoderm differentiation cultures. B. QRT-PCR gene expression analysis of H9 ES cells 72 hrs after *OCT4* siRNA knockdown (i) pluripotency genes, (ii) primitive streak genes comparing to D1 differentiation cells and (iii) definitive endoderm genes comparing to D3 differentiation cells. C. QRT-PCR gene expression analysis of endoderm differentiation cultures at (i) D0, (ii) D1 and (iii) D3 after *OCT4* knockdown. D. A second *OCT4* siRNA (#2) can also knockdown *OCT4* and impair endoderm differentiation. Flow cytometry analysis on OCT4 and endodermal markers in differentiation cultures as described in figure 3. E. ChIP-qPCR analysis of anti-EZH2 and anti-H3K27me3 at *GSC* regulatory regions after *OCT4* knockdown at D1 of endoderm differentiation cultures. Sites used for ChIP-qPCR are indicated in kilobases (K) from the transcription start site. Data shown are from three independent experiments. Statistical significance indicated as * for $p < 0.05$. F. EZH2 and SUZ12 intracellular flow cytometric analysis on H9 D1 OCT4 knockdown cells.





D.

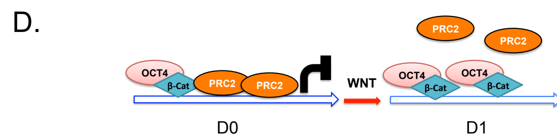


Figure S3. Analysis of *OCT4* knockdown in CHOPWT2.1 iPS cells. CHOPWT2.1 iPS cells were transfected with siRNA against *OCT4* or a control scramble siRNA construct 18 hours prior to initiation of endoderm differentiation as described in Figure 1. A. Flow cytometry analysis of SOX2, NANOG and OCT4 18 hrs after *OCT4* siRNA transfection. B. Flow cytometry analysis of CXCR4, FOXA1, and SOX17 on D3 differentiation cultures treated with *OCT4* or scramble siRNA. C. (i) Anti-SUZ12 and (ii) anti-H3K27me3 ChIP-qPCR of D1 differentiation cultures treated with OCT4 or scramble siRNA on primitive streak genes (*T* and *EOMES*) and endodermal genes (*FOXA2* and *SOX17*). Sites used for ChIP-qPCR are indicated in kilobases (K) from the transcription start site. Data shown are from of three independent experiments. Statistical significance represented as * for $p < 0.05$. D. Working model of how OCT4 and β-Catenin cooperate to evict the PRC2 complex and allow endodermal gene expression.

Supplemental Tables

Table S1. siRNA Sequence

Sequence Name	Sequence
OCT4 #1 Anti-Sense	rUrArC rUrGrG rUrUrC rGrCrU rUrUrC rUrCrU rUrUrC rGrGrG rCrCrU
OCT4 #1 Sense	rGrCrC rCrGrA rArArG rArGrA rArArG rCrGrA rArCrC rArGT A
OCT4 #2 Anti-Sense	rUrCrC rArGrG rUrUrG rCrCrU rCrUrC rArCrU rCrGrG rUrUrC rUrCrG
OCT4 #2 Sense	rArGrA rArCrC rGrArG rUrGrA rGrArG rGrCrA rArCrC rUrGG A
Scramble Antisense	rArUrG rArUrA rUrArG rArCrG rUrUrG rUrGrG rCrUrG rUrUrG rUrArG
Scramble Sense	rArCrA rArCrA rGrCrC rArCrA rArCrG rUrCrU rArUrA rUrCA T

Table S2. Primers for ChIP

NAME	Forward	Reverse
EOMES -7K	GGA GAA GGC ACC CTT AAC TGG ATG T	GAT CTT GCT CTG CAC TTG CTC TGT
GSC -3.5K	CCA GTC TCA AAG CTA AGT GCT CAA ACC	TCC ACC CAC CCA ACC TCA C
GSC P	ACA GAG CAG GTG AAG AGA GAG CAA	ACA GAG CAG GTG AAG AGA GAG CAA
FOXA2 +1.2K	ACCTCGGGCTCTGCATAGTA	TTTAAACTGCCATGCACTCG
OLIGO1 P	GGCACAAGCAGCCAATGAACAC	CTTTAAACCCGGCTTGGGAACCT
PAX6 P	GCGGACTCACCTTTATGAGG	CCATTGTGGTCTTCAAGCAA
SOX17 -1.5K	CCCTAGCCAAGGCTTCACAGATACTT	CCGTCTGTCCAGTCTTGCTTATTAGTGG
SOX17 -3.6K	AGCGGTGTACTCGGCTCTTA	GCCACATGTAAAGCAGCAAA
T -1.5K	CAGGTGGTCCACTCGGTACT	AGGGAAGGTGGATCTCAGGT
T -5K	ACCAGAGAGTCCAGGGAGGT	GGCTCTCTTTCCTCATGTGC

Table S3. Primers for RT-PCR

NAME	Forward	Reverse
CER1	TCCCATACCTCCTGCTCTCACTGTTT	TGTGTCCATCTTCATGCTCCGTCTTC
CYCLO	GAA GAG TGC GAT CAA GAA CCC ATG AC	GTC TCT CCT CCT TCT CCT CCT ATC TTT ACT T
EOMES	TAT TGT CGG CTT TGC CAC AGG TCA	ACT CAA TCC CAC TGC CCA CTA CAA
EZH2	AGG AGT GTA AGC TTT GCT CTC TCT GA	CAC CGA ACA GCA GCT CCC A
FOXA2	GCA CCT TCA GGA AAC AGT CGT TGA	ACT CGT ACA TCT CGC TCA TCA CCA
FOXA1	ACT ACT CCT TCA ACC ACC CGT TCT	TAT TGC AGT GCCTGT TCG TAT GCCTTG
GSC	GGT ACT TGG TCT CCT GGA AGA GGT T	ATG CTG CCC TAC ATG AAC GTG G
MIXL1	TAG ATG TGA ACT GCC TGC CCG AA	TGT TCC TCC CAT GAG TCC AGC TTT
NANOG	CCTGAAGACGTGTGAAGATGAG	GCTGATTAGGCTCCAACCATAC
OCT4	TTC GGG CAC TGC AGG AAC AAA TTC	TAT GCA AAG CAG AAA CCC TCG TGC
SOX17	AGG AAA TCC TCA GAC TCC TGG GTT	CCC AAA CTG TTC AAG TGG CAG ACA
SOX2	ATG ACC AGCTCG CAG ACCTAC A	GGA CTT GAC CAC CGA ACC CA
SUZ12	TGT CGA AAC TTC ATG CTT CAT CTA GTC	TGT TCT TCA GTT ATT TCT TTT GCA
TBP	TTG CTG AGA AGA GTG TGC TGG AGA TG	CGT AAG GTG GCA GGC TGT TGT T

Supplemental Experimental Procedures

ChIP-qPCR Assay

ChIP assays were carried out as previously described (Tuteja et al., 2008). Briefly, cells were crosslinked with 1% formaldehyde. Ideal length of chromatin fragments (300 – 500 bp) was achieved by sonicating with Misonix3000 with a micro tip. Chromatin was immunoprecipitated with following antibodies: H3K27me3 (Millipore, 07-449), OCT4 (Santa Cruz, sc-8628), EZH2 (Active Motif, 39875), SUZ12 (Abcam, ab12073). Primer sequences are listed in Table S2.

Gene Ontology Analysis

The hES OCT4 ChIP-seq dataset (GSE21916) (Marson et al., 2008) and H3K4me3 and H3K27me3 ChIP-seq dataset (GSE23455) (Guenther et al., 2010) were used. Co-occupancy of OCT4 and bivalent domains was obtained by intersecting 2 datasets using GALAXY (Goecks et al., 2010). Then, gene ontology term enrichment was computed by feeding GREAT (McLean et al., 2010) with OCT4 binding bivalent domain position information.

Flow Cytometry and Immunofluorescence

Flow cytometry was carried out as previously described (Gadue et al., 2006). Briefly, for cell surface markers, cells were resuspended in FACS buffer and stained with anti-CXCR-4-PE (Invitrogen, MXCXCR404) and anti-CD117-APC (Invitrogen, CD11705). For intracellular markers, cells were fixed with 1.6% PFA and permeablized with saponin buffer (Biolegend). Antibodies used for intracellular staining were anti-OCT4 (Santa Cruz, sc-5279), anti-SOX2 (BD MAB2018), anti-β-Catenin (BD, 610154), anti-FOXA1 (Santa Cruz, sc-101058) and biotinylated anti-SOX17 (R&D, BAF1924). Cells were acquired with BD FACS Canto II (BD Sciences). Data were analyzed with FlowJo software.
For immunofluorescence staining, cells were replated onto matrigel-coated glass cover slips. Cells were harvest at D0, D1 and D2 of differentiation and fixed with 1% PFA. Cells were permeablized with 0.1% Triton-X 100. Antibodies used for immunofluorescence staining were anti-OCT4 (Santa Cruz, sc-5279), anti-β-Catenin (BD, 610154). Stained cells were mounted with ProLong Gold anti-fade mountant with DAPI (Invitrogen). The stained cells were visualized using a fluorescence microscope (Leica DMI 4000B) and images captured and analyzed using the Leica Application Suite software. Distribution of fluorescence intensity across a single representative cells was measured and plotted, demonstrating nuclear localization of ABC at D1.

Reverse Transcription and Real-time Quantitative PCR (qRT-PCT)

RNA was extracted using RNeasy Micro RNA Extraction kit (Qiangen). Reverse transcription was performed using SuperScript III (Invitrogen), as per manufacturer instructions. Quantitative PCR was performed on a Light Cycler 480 (Roche) using LightCycler SYBR Green Mater Mix I (Roche) and normalized to the housekeeping genes TBP or CYCLOPHILIN. Data shown are representative of three independent experiments. Statistical significance indicated as * for $p < 0.05$. Primer sequences are listed in Table S3.

Western Blot and Immunoprecipitation

Western blot and immunoprecipitation were carried out as previously described (Kelly et al., 2011). For immunoprecipitation, 20 million of D0 hESCs or D1 cells were treated with hypotonic buffer for 10 min on ice, then, nuclei protein was extracted with high salt buffer. 400 μg of nuclei protein was subjected to immunoprecipitation with anti-OCT4 antibody (Santa Cruz, sc-8628). Eluted material was blotted with either anti-OCT4 antibody (Santa Cruz, sc-8628), anti-totalβ-catenin antibody (BD) or anti-activatedβ-catenin antibody (Life Sciences, 712700).

Statistics

All experiments were performed at least three times and statistical significance was calculated by Student's t test. For ChIP qPCR results, *p* value was calculated by Student's T test. Error bars represent SEM from 3 biological replicates. All experiments were performed at least three times. Results with statistic significance ($p<0.05$) were labeled with asterisk (*).

Supplemental References

Goecks, J., Nekrutenko, A., Taylor, J., and Galaxy, T. (2010). Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. Genome biology *11*, R86.

Guenther, M.G., Frampton, G.M., Soldner, F., Hockemeyer, D., Mitalipova, M., Jaenisch, R., and Young, R.A. (2010). Chromatin structure and gene expression programs of human embryonic and induced pluripotent stem cells. Cell Stem Cell *7*, 249-257.

Kelly, K.F., Ng, D.Y., Jayakumaran, G., Wood, G.A., Koide, H., and Doble, B.W. (2011). beta-catenin enhances Oct-4 activity and reinforces pluripotency through a TCF-independent mechanism. Cell Stem Cell *8*, 214-227.

Marson, A., Levine, S.S., Cole, M.F., Frampton, G.M., Brambrink, T., Johnstone, S., Guenther, M.G., Johnston, W.K., Wernig, M., Newman, J., *et al.* (2008). Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. Cell *134*, 521-533.

McLean, C.Y., Bristor, D., Hiller, M., Clarke, S.L., Schaar, B.T., Lowe, C.B., Wenger, A.M., and Bejerano, G. (2010). GREAT improves functional interpretation of cis-regulatory regions. Nature biotechnology *28*, 495-501.