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Zic3 Is Required for Maintenance of Pluripotency in Embryonic Stem Cells[D]

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Embryonic stem (ES) cell pluripotency is dependent upon sustained expression of the key transcriptional regulators Oct4, Nanog, and Sox2. Dissection of the regulatory networks downstream of these transcription factors has provided critical insight into the molecular mechanisms that regulate ES cell pluripotency and early differentiation. Here we describe a role for Zic3, a member of the Gli family of zinc finger transcription factors, in the maintenance of pluripotency in ES cells. We show that Zic3 is expressed in ES cells and that this expression is repressed upon differentiation. The expression of Zic3 in pluripotent ES cells is also directly regulated by Oct4, Sox2, and Nanog. Targeted repression of Zic3 in human and mouse ES cells by RNA interference–induced expression of several markers of the endodermal lineage. Notably, the expression of Nanog, a key pluripotency regulator and repressor of extraembryonic endoderm specification in ES cells, was significantly reduced in Zic3 knockdown cells. This suggests that Zic3 may prevent endodermal marker expression through Nanog-regulated pathways. Thus our results extend the ES cell transcriptional network beyond Oct4, Nanog, and Sox2, and further establish that Zic3 plays an important role in the maintenance of pluripotency by preventing endodermal lineage specification in embryonic stem cells.

INTRODUCTION

The transcription factors Oct4, Nanog, and Sox2 are key regulatory players in embryonic stem (ES) cell biology. These core factors contribute to the hallmark characteristics of ES cells by 1) activation of target genes that encode pluripotency and self-renewal mechanisms and 2) repression of signaling pathways that promote differentiation (Orkin, 2005). In ES cells Oct4, Nanog, and Sox2 co-occupy promoters of hundreds of genes that are both expressed and repressed in the pluripotent state (Boyer *et al.*, 2005; Loh *et al.*, 2006). This suggests complex regulatory circuitry in which Oct4, Nanog, and Sox2 collectively and uniquely regulate downstream genes to control ES cell differentiation. However, it remains unclear what are the downstream effectors of these transcription factors that contribute to maintaining the pluripotent status of ES cells. It also not understood how these "master regulators" of pluripotency are involved in controlling lineage-specific differentiation of ES cells. It is therefore useful to elucidate the transcriptional networks surrounding Oct4, Nanog, and Sox2, where detailed knowledge of these pathways remain key to harnessing the potential to direct differentiation of ES cells into therapeutically useful cell types.

To expand our understanding of the transcriptional networks that control stem cell differentiation, we have looked at transcription factors whose expression is directly regulated by Oct4, Nanog, and Sox2. We have identified Zic3 (Zinc finger protein of the cerebellum 3) as a transcription factor of interest for two main reasons. First, Oct4, Nanog, and Sox2 binding have been mapped to the Zic3 promoter regions in ES cells (Boyer *et al.*, 2005; Loh *et al.*, 2006), implying that these key factors may regulate Zic3 expression. The overlap between mouse and human ES cells further highlights the significance of Zic3 and suggests possible conservation of the gene's pathways between the two species. Second, Zic3 demonstrates differential gene expression between the pluripotent and early differentiation phases, where its expression is higher in the pluripotent state (Brandenberger *et al.*, 2004; Wei *et al.*, 2005). The changes in gene expression between these states suggest a potential role for Zic3 in controlling differentiation of mouse and human ES cells.

Zic3 belongs to the GLI superfamily of transcription factors and is a vertebrate homologue of the *Drosophila* pair-rule gene odd-paired (*opa*; Aruga *et al.*, 1996a). The five known mammalian Zic genes (Zic1-5) encode five tandem C_2H_2 zinc finger domains that are highly conserved across species (Herman and El-Hodiri, 2002; Grinberg and Millen, 2005). Although the expression of Zic3 is restricted to the cerebellum of adult mammals, dynamic patterns of expression have been observed during embryonic development in mouse (Herman and El-Hodiri, 2002), *Xenopus* (Nakata *et al.*,

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Abbreviations used: ES, embryonic stem; RA, retinoic acid; RNAi, RNA interference; ChIP, chromatin immunoprecipitation; AVE, anterior visceral endoderm.

1997, 1998), chick (Warner *et al.*, 2003), and zebrafish (Grinblat and Sive, 2001). The expression of Zic3 in the embryonic ectoderm and mesoderm during gastrulation (Kitaguchi *et al.*, 2002; Elms *et al.*, 2004), and throughout the tailbud, retina and limb bud during neurulation and organogenesis (Herman and El-Hodiri, 2002; Orkin, 2005), suggests an important role for this transcription factor in embryonic ectoderm and mesoderm development. This is further supported by molecular pathways in which Zic3 has been implicated. For example the mesoderm-associated gene Brachyury induces Zic3 expression in *Xenopus* (Kitaguchi *et al.*, 2002), and the embryonic patterning gene Nodal is regulated by Zic3 during gastrulation through interaction with an upstream enhancer region in mouse and *Xenopus* embryos (Ware *et al.*, 2006a). In ectodermal development, Zic3 is a potent inducer of *Xenopus* proneural and neural crest genes (Nakata *et al.*, 1997) and is induced directly downstream of transcription factors Pbx1b and Meis1 in the *Xenopus* ectoderm (Maeda *et al.*, 2002; Kelly *et al.*, 2006).

Zic3 mutations are associated with X-linked heterotaxy, a disorder characterized by disruptions in embryonic laterality and midline developmental field defect (Gebbia *et al.*, 1997). In Zic3 mutant organisms s*itus ambiguus* is frequently observed, encompassing failure in lateralization of internal organs, mirror-image inversions, and left-right isomerism (Aylsworth, 2001). Several mutations have been identified in humans that render the Zic3 protein unstable and absent in cells or incapable of nuclear localization where its transcriptional effect is exerted (Gebbia *et al.*, 1997; Ware *et al.*, 2004).

Consistent with its expression in the involuting mesoderm and presumptive neural plate during gastrulation, Zic3 is involved in regulating left–right asymmetry and neural tube development. Zic3-null mice exhibit a wide spectrum of phenotypes. Fifty percent of null mice succumb to embryonic lethality over different gestational stages, and 30% to perinatal lethality as a result of congenital heart defects, pulmonary isomerism, and defects in the CNS (Purandare *et al.*, 2002). The earliest and most profound Zic3-null defects have been attributed to failure in establishment of the anterior-posterior axis by the anterior visceral endoderm (AVE) before gastrulation (Ware *et al.*, 2006b). In less severely affected embryos, abnormalities are observed at gastrulation in the distribution and accumulation of excess mesoderm tissue. Taken together, the defects in embryonic lethal mice demonstrate a key role for Zic3 in early embryonic patterning that encompasses anterior visceral endoderm formation, initiation of gastrulation, and primitive streak morphogenesis (Ware *et al.*, 2006b).

The varying degrees of severity in failure to complete gastrulation displayed by Zic3 null mice may perhaps be attributed to compensatory mechanisms in developing embryos, as indicated by the distinct and partially overlapping expression patterns exhibited by members of the Zic gene family (Nagai *et al.*, 1997; Elms *et al.*, 2004). It is important to note that Zic3 shares overall 64 and 59% homology with Zic1 and Zic2, respectively, and this homology increases to 91% within the zinc finger domain. Thus members of Zic family are strong candidates for redundancy in molecular signaling owing to the high degree of homology and overlapping expression observed among the members of this family.

Although Zic3 expression has been implicated in embryonic development, still lacking is a detailed understanding of what regulates Zic3 expression and what the downstream effectors of Zic3 are. The Zic3 gene has been identified as a target of Oct4, Nanog, and Sox2 in ES cells (Boyer *et al.*, 2005; Loh *et al.*, 2006), and Zic3 is preferentially expressed in pluripotent state (Brandenberger *et al.*, 2004; Wei *et al.*, 2005). Questions arising from these data are as follows: 1) How do Oct4, Nanog, and Sox2 interact with the Zic3 regulatory region, and what results from this interaction and, 2) what

Table 1. List of marker genes used to assess lineage development in ES cells

Gene symbol	Description	Lineage
Sox17	SRY-box containing gene 17	Endoderm
PDGFRA	Platelet-derived growth factor receptor, alpha	Endoderm
Gata4	GATA binding protein 4	Endoderm
Gata6	GATA binding protein 6	Endoderm
Foxa2	Forkhead box A2	Endoderm
GSC	Goosecoid	Mesendoderm
Nodal	Nodal	Mesendoderm
MixL1	Mix1 homeobox-like 1	Mesendoderm
Hand1	Heart and neural crest derivatives expressed 1	Mesoderm
Nkx2.5	NK2 transcription factor related, locus 5	Mesoderm
Gata2	GATA binding protein 2	Mesoderm
Nestin	Nestin	Ectoderm
GFAP	Glial fibrillary acidic protein	Ectoderm
Pax6	Paired box gene 6	Ectoderm
TDGF1	Teratocarcinoma-derived growth factor/Cripto	Ectoderm
Sox1	SRY-box containing gene 1	Ectoderm
REST	RE1-silencing transcription factor	Ectoderm
CoREST	REST Co-repressor 1	Ectoderm
FGF5	Fibroblast growth factor 5	Ectoderm
BMP4	Bone morphogenetic protein 4	Trophectoderm
CDX2	Caudal type homeobox 2	Trophectoderm
DKK3	Dickkopf homolog 3	Wnt pathway
Gsk3beta	Glycogen synthase kinase 3 beta	Wnt pathway

role does Zic3 play in the embryonic stem cell? We have addressed these questions using the loss-of-function approach for Zic3 and the key regulatory genes in ES cells. In this study, we examined the function of Zic3 as a regulatory target of Oct4, Nanog, and Sox2 in ES cells. We report that Zic3 shares significant overlap with the Oct4, Nanog, and Sox2 transcriptional networks and is important in maintaining ES cell pluripotency by preventing differentiation of cells into endodermal lineages. Thus our results extend the current knowledge of the ES cell transcriptional circuitry beyond Oct4, Nanog, and Sox2.

MATERIALS AND METHODS

ES Cell Maintenance

Feeder-free E14 Mouse ES cells were maintained on 0.1% gelatin-coated dishes in E14 proliferative medium containing DMEM/15% ES FBS (Invitrogen, Carlsbad, CA), 0.1 mM MEM nonessential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen), and Chinese hamster ovary-Leukaemia Inhibitory Factor (CHO-LIF) (1000 U/ml). Feeder-free undifferentiated HuES9 human ES cells were maintained on matrigel-coated dishes in conditioned medium containing knockout DMEM/10% serum replacement (Invitrogen), 0.1 mM MEM nonessential amino acids (Invitrogen), 1 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen), 8% plasmanate (National University Hospital Pharmacy, Singapore), 12 ng/ml LIF, and 10 ng/ml human recombinant basic fibroblast growth factor (bFGF; Invitrogen). Conditioned medium was obtained by culturing mouse embryonic fibroblast (MEF) cells with HuES9 media. The medium was collected at 24 h intervals, filter sterilized, and further supplemented with 8 ng/ml bFGF for HuES9 cell culture.

RNA Interference and Establishment of Clonal Knockdown Lines

Small Interfering RNA (siRNA) Experiments. RNA interference (RNAi) experiments were performed with Dharmacon siGENOME SMARTpool reagents (Boulder, CO) against human or mouse Zic3. The Dharmacon si*CONTROL* nontargeting siRNA pool was used as a negative control. Mouse ES cells were transfected according to manufacturer's instructions in 12-well plates at a density of 2×10^5 cells per well. Retransfections were performed on preadherent cells at 48-h intervals, and RNA expression analysis was performed on samples from day 5. Human ES cells were transfected in 12-well plates with 2×10^5 cells, in suspension, per well. Subsequent retransfections were performed on adherent cells at 24-h intervals and RNA was harvested for analysis at day 5.

Short Hairpin RNA (shRNA) Experiments. The Oct4 and Nanog RNAi experiments were previously published (Loh *et al.*, 2006). The Zic3 shRNA construct was designed as described (Chew *et al.*, 2005) with a target sequence of 5′-GAATTCGAAGGCTGTGACA-3′. E14 cells in six-well plates were transfected with 2.0 μg pSUPERpuro vector or Zic3-pSUPER.puro (OligoEngine, Seattle, WA) at a density of 4×10^5 cells per well. Puromycin selection was introduced 1 d after transfection at 1.0 μg/ml and was maintained for 3 d before RNA isolation. ES cells were maintained in proliferative medium at all times.

Clonal Zic3 knockdown lines were established by transfection of shRNA constructs as described above. The Zic3 knockdown and vector control colonies were picked after 7 d of puromycin selection (1.0 μg/ml). Colonies were dissociated into single-cell suspensions by treatment with 0.05% Trypsin (Invitrogen) and plated on puromycin-resistant mitomycin-inactivated DR4 MEFs (ATCC, Manassas, VA). In total, 15 Zic3 clonal knockdown and 7 vector control lines were established and maintained under constant puromycin selection. The lines analyzed in this article were maintained feeder-free in ES cell proliferative media on 0.1% gelatin-coated dishes over a period of eight passages.

Secondary ES Colony-replating Assay

ES cells were transfected with Zic3- or empty pSUPER shRNA constructs and selected 24 h later with puromycin at 1.0 μg/ml over 4 d. At the end of 4 d few cells remained in the untransfected control wells indicating that selection was effective. The surviving cells were trypsinized and resuspended in E14 medium without LIF. Ten thousand or 20,000 cells were plated onto mouse feeder layers in six-well plates for secondary ES cell-colony formation. After 7 d, emerging colonies were stained with the Wright-Giemsa (Sigma, St. Louis, MO) stain. The extent of differentiated colonies was defined as the percentage of unstained colonies out of the total number of colonies in the well.

RNAi Rescue Experiments

The Zic3 open reading frame (ORF; NM_009575) was cloned from reverse-transcribed cDNA from mouse embryonic stem cells, using the primers indicated in Supplementary Table 1A. The PCR product was subsequently cloned into a vector driven by the CAG promoter. The RNAi-immune Zic3 ORF R3M (Supplementary Figure 1) was generated from this template using site-specific mutagenesis. To perform the rescue experiments, 4×10^5 mouse ES cells were seeded per well in six-well plates and transfected according to the scheme in Supplementary Table 1B. Hygromycin selection (1.0 μg/ml) was introduced 1 d after transfection.

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

To minimize genomic DNA contamination, RNA was extracted with TriZol reagent (Invitrogen) and further purified with the RNeasy minikit (Qiagen, Chatsworth, CA). cDNA was synthesized with 1.0 μg total RNA using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). For each qPCR reaction, cDNA samples diluted 10 times in water were mixed with 5.0 μl TaqMan Universal PCR Master Mix reagent (Applied Biosystems) and 0.5 μl of a single TaqMan probe from the following list: Zic3, Oct4, Nanog, Sox2, or the lineage markers in Table 1 (20× TaqMan Gene Expression

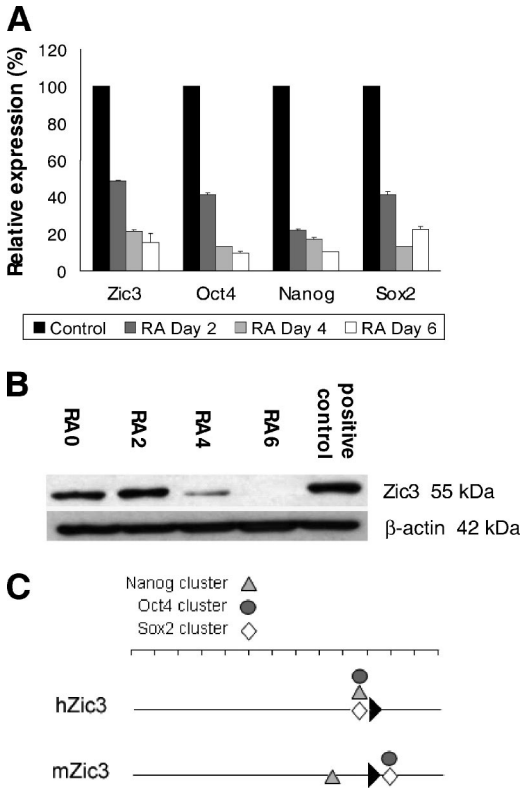

Figure 1. A profile of Zic3 expression in differentiating E14 cells. (A) Real-time PCR analysis of differentiation induced by retinoic acid. Samples were assayed at 2-d intervals (untreated control, and treated samples day 2, day 4, and day 6). Mean levels ± SE are expressed as percentages relative to undifferentiated E14 cells (100%). The assays were conducted in duplicate and normalized to β-actin control. (B) Verification of Zic3 protein expression during the process of RA differentiation. (C) A summary of ChIP mapping of Oct4, Nanog, and Sox2 binding sites on the Zic3 regulatory regions in mouse ES cells (Loh *et al.*, 2006; Sox2, Ng, unpublished data) and human ES cells (Boyer *et al.*, 2005). We examined transcription factor binding sites within 100 kb up- and downstream of the Zic3 coding region. In human ES cells, Oct4, Nanog, and Sox2 binding sites were located within 3.5 kb upstream of the Zic3 transcription start site, whereas in mouse ES cells, the Nanog binding site was found within 18.5 kb upstream, and the Oct4 and Sox2 binding sites were within 9.5 kb downstream of the gene, respectively (Loh *et al.*, 2006). Each unit on the scale represents 10 kb.

Assay reagents; Applied Biosystems) with a final volume of 10 μl. Quantitative real-time PCR analysis was conducted in 384-well clear optical reaction plate (Applied Biosystems) on the ABI Prism 7900 machine (Columbia, MD).

Western Blots and Immunocytochemistry

Zic3 protein detection was performed with goat-anti-Zic3 antibody (1:800 dilution; C-12, Santa Cruz Biotechnology, Santa Cruz, CA) and donkey anti-goat horseradish peroxidase (HRP; 1:5000; Santa Cruz Biotechnology). Loading consistency was determined with mouse anti-β-actin (1:3000; Invitrogen) and goat anti-mouse HRP (1:5000; Santa Cruz Biotechnology). For immunocytochemistry, cells were seeded at a density of 1.0×10^5 cells per well on fibronectin-coated chamber slides, fixed in 4% paraformaldehyde, and permeabilized with 0.3% Triton X-100. Blocking was performed with 5% fetal bovine serum and 1% bovine serum albumin in PBS solution for 30 min. Cells were stained with the following primary antibodies (1:100): goat or mouse anti-Oct4 (Santa Cruz Biotechnology, N-19 and C-10, respectively), rabbit-anti-Nanog (Chemicon, Temecula, CA; AB5731), goat anti-FoxA2 (M-20, Santa Cruz Biotechnology), goat-anti-Gata6 (C-20, Santa Cruz Biotechnology), or mouse anti-CD140a (PDGFRA; eBioscience, San Diego, CA; 16-1401). This was followed by the appropriate secondary antibodies detecting mouse or goat IgG Alexa Fluor 488 (Molecular Probes, Eugene, OR; 1:500) for Oct4 staining, rabbit IgG Alexa Fluor 594 (Molecular Probes; 1:500) for Nanog staining, or Qdot 655 anti-goat or anti-mouse antibodies (Molecular Probes) for FoxA2, Gata6, and PDGFRA staining (1:150) according to the manufacturer's protocol. Images were captured with the Zeiss LSM 5 Duo inverted confocal microscope (Zeiss, Thornwood, NY).

Luciferase Reporter Construct and Assays

The 300-base pair Zic3 enhancer region containing the Nanog-binding site was cloned from mouse genomic DNA. The primers used were as follows: forward, 5′ ATATAacgcgtTTAGAGGTCAAACCAT-3′ and reverse, 5′-TATATagatctTAGTAGTCAAACTGGATT-3′ with restriction sites indicated in lower case letters. The PCR fragment was digested with MluI and BglII and cloned into the pGL3-Basic vector (Promega, Madison, WI) containing a basal promoter comprising the 500-bp region immediately upstream of the mouse Oct4 gene. The following constructs were transfected into cells 2.5×10^4 cells in 96-well plates for the luciferase assay: 100 ng firefly luciferase reporter, 1.0 ng of the *Renilla* luciferase vector, pRL-SV40 plasmid normalization control, and 250 ng of the respective knock-down construct. Puromycin selection (1.0 μg/ml) was introduced 20 h after transfection and cultured for 2 d. Luciferase activity measured using the Dual Luciferase System (Promega) in a Centro LB960 96-well luminometer (Berthold Technologies, Natick, MA).

RESULTS

Zic3 Expression Is Associated with ES Cell Pluripotency

Comprehensive expression profiling of mouse and human ES cells has identified numerous genes that are expressed in undifferentiated cells and quickly repressed upon differentiation (Brandenberger *et al.*, 2004; Wei *et al.*, 2005). Among these genes are transcription factors Oct4, Nanog, and Sox2, which are required to maintain pluripotency of ES cells. Zic3, a zinc-finger transcription factor, was also found to be expressed in undifferentiated ES and suppressed in differentiated cells, and thus, may play a role in regulating ES cell differentiation. We assayed the expression of Zic3 in mouse ES cells induced to differentiate over 6 d by addition of retinoic acid (RA; Figure 1A). Similar to the trends observed for Oct4, Nanog, and Sox2 genes, Zic3 transcript levels decreased between 1.5- and 10-fold for each 2-d interval (D2, D4, and D6), relative to the undifferentiated control. Zic3

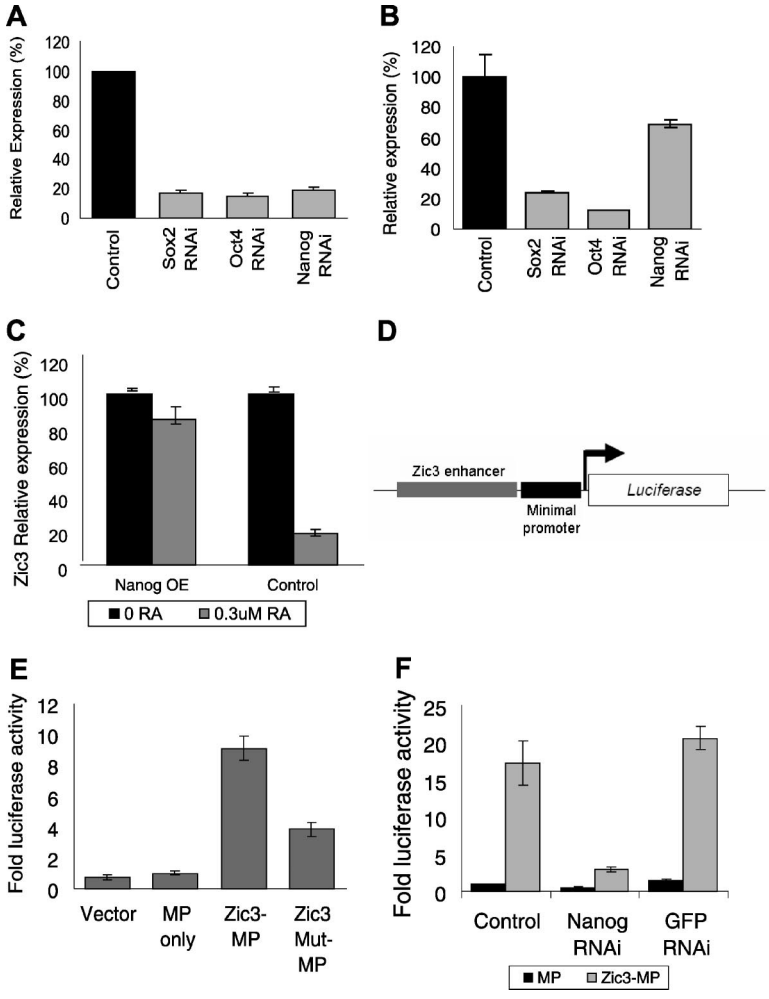

Figure 2. Oct4, Sox2, and Nanog regulate Zic3 expression. (A) Changes in endogenous gene expression levels of Oct4, Nanog, and Sox2 after gene-specific RNAi and (B) corresponding changes in endogenous *Zic3* gene levels. cDNAs were prepared from the RNAi knockdown ES cells and analyzed using real-time PCR. The levels of the transcripts were normalized against values derived from control RNAi-transfected ES cells (100%). (C) Changes in ES cell endogenous Zic3 gene level after Nanog overexpression with RA induced differentiation. Nanog overexpression cell line and control cell line were treated with no RA or 0.3 μM RA for 2 d. Transcript levels of 0.3 μM RA-treated sample were normalized against no RA treatment sample. (D) Diagram of the construct with putative Zic3 enhancer region fused upstream of a minimal Pou5f1 promoter and firefly luciferase gene. (E) The effects of luciferase activity in deletion of the putative Nanog binding site on Zic3 enhancer were tested by transfecting into ES cells. Activity were measured relative to the minimal promoter only (MP) construct without the Nanog enhancer. (F) Effects of Nanog RNAi on Zic3 enhancer activity were tested by cotransfecting the Nanog RNAi with the reporter construct into ES cells and luciferase activity measured. Activity were normalized against the Control RNAi with mOct4 promoter-only construct. An RNAi targeting the GFP sequence was used as a non-specific control.

RNA levels were also significantly decreased in mouse ES cells differentiated by treatment with HMBA (hexamethylene bisacetamide) or dimethyl sulfoxide, and also by aggregation into embryoid bodies (data not shown). The decrease in Zic3 mRNA correlated with a comparable decrease in protein expression (Figure 1B). Thus, Zic3 gene expression is associated with the mouse ES pluripotent state and its expression decreases as cells differentiate.

Chromatin immunoprecipitation experiments in both mouse and human ES cells have identified binding sites for the transcription factors Oct4, Nanog, and Sox2 at the Zic3 gene locus (Figure 1C; Boyer *et al.*, 2005; Loh *et al.*, 2006). The binding of these transcription factors, which are demonstrated regulators of pluripotency, suggests that Zic3 is a direct target for regulation by these TFs and may play a role in regulating ES cell differentiation.

Regulation of Zic3 by Oct4, Sox2, and Nanog

To further validate that Oct4, Sox2, and Nanog regulate Zic3 expression, we performed gene expression knockdown experiments in mouse ES cells using RNA interference. Mouse ES cells were thrice transfected with gene-specific siRNAs against Oct4, Sox2, and Nanog on alternate days to achieve 80–90% reduction in expression of the targeted gene (Figure 2A). Down-regulation of Oct4 and Sox2 reduced the level of endogenous Zic3 to <25%, whereas Nanog RNAi reduced the level of Zic3 to 70% (Figure 2B). These data indicate that Zic3 expression is regulated by Oct4, Sox2, and Nanog.

It has been shown that Nanog-overexpressing ES cells are resistant to differentiation induced by LIF withdrawal and RA addition (Chambers and Smith, 2004). As the endogenous levels of Zic3 decreased in the presence of RA-induced differentiation (Figure 1), we were interested in determining if Nanog overexpression would sustain Zic3 levels under RA treatment. ES cells were stably transfected with a construct that expresses Nanog from a constitutively active promoter. The Nanog-expressing cells and cells transfected with empty vector were treated for 2 d with 0.3 μM RA. Vector-only control cells showed a decrease in Zic3 RNA levels typical of RA-induced differentiation. In contrast, mouse ES cells overexpressing Nanog sustained the level of Zic3 at greater than 80%, relative to the control ES cell line (Figure 2C). Thus, overexpression and knockdown of Nanog in ES cells results in an increase and decrease, respectively, of Zic3, suggesting that Zic3 expression is regulated by Nanog, perhaps directly or indirectly.

Our previous study identified a Nanog binding site in the enhancer region, 16.4 kb upstream of the transcription start site, of the Zic3 gene (Loh *et al.*, 2006). As this DNA region was available for further study in our lab, we sought to determine if Zic3 expression was directly regulated by Nanog. We fused the 292-base pair portion of the Zic3 enhancer that contains the Nanog-binding site upstream of a minimal Pou5f1 promoter driving the firefly luciferase gene (Figure 2D). The minimal promoter was weakly active in ES cells, whereas activity of the Zic3 enhancer region linked to the minimal promoter was ninefold up-regulated as quantified by luciferase (Figure 2E). When the sequences of this putative Nanog binding site were deleted from the Zic3 enhancer the corresponding reporter activity decreased (Figure 2E). We then transfected Nanog RNAi together with the wild-type reporter construct and showed that the activity of the Zic3 enhancer decreased fourfold relative to the controls (Figure 2F). Collectively, our data show that Zic3 expression is directly regulated by Nanog and thus, may be a downstream effector in controlling ES cell differentiation.

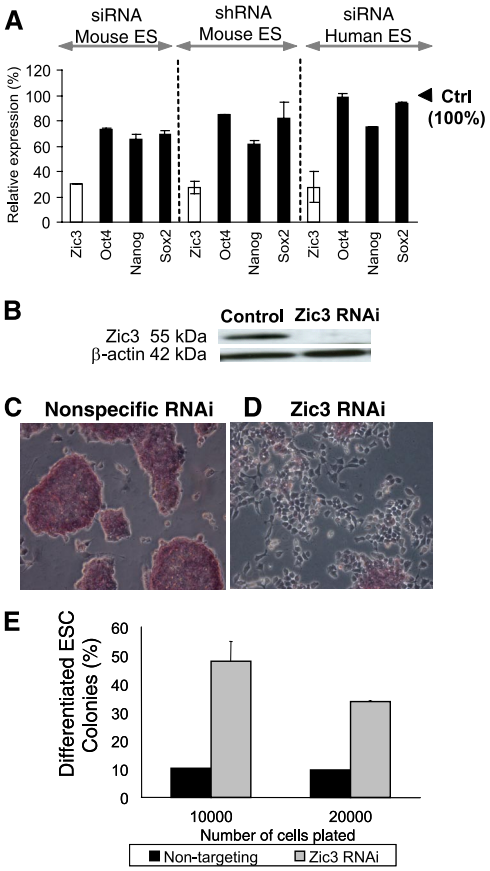

Figure 3. Effect of Zic3 RNAi on endogenous Oct4, Nanog, and Sox2 levels. (A) Zic3 levels were depleted by RNAi using siRNA and shRNA in mouse E14 cells and siRNA in human HuES9 cells. RNA was harvested between 4 and 5 d of transfection and transcript levels assayed by real-time PCR. Shown in this figure are the levels of Zic3 transcript and the corresponding changes in Oct4, Nanog, and Sox2 expression. Mean values ± SE are plotted as percentages relative to the nontargeting control (100%). The samples were assayed in duplicate and normalized to endogenous β-actin. (B) Corresponding decrease in protein levels after Zic3 RNAi treatment. The Zic3 protein species was depleted in the Zic3 RNAi sample, whereas β-actin protein levels remained high in the control. β-actin protein was used as a loading control. (C and D) Alkaline phosphatase staining revealed that the extent of differentiation in Zic3 RNAi-treated cells was greater than mock-transfected cells. (E) Secondary replating assays were used to quantitate the extent of differentiation in Zic3 RNAi cells. A 3- to 5-fold increase in differentiated colonies were observed with Zic3 RNAi relative to mock-transfected control.

Effect of Zic3 Depletion on ES Cell Differentiation

To investigate the role of Zic3 in ES cells, we used RNAi to achieve knockdown of gene expression. Both the siRNA and shRNA methods resulted in a 70% reduction of Zic3 transcript levels relative to the nontargeting controls (Figure 3A). Zic3 protein levels reflect this decrease in gene expression after Zic3 RNAi treatment, whereas protein expression remained high in vector-only–treated cells (Figure 3B).

Zic3 RNAi transfections resulted in a marked decrease in pluripotent colonies that stained for alkaline phosphatase (AP) relative to the mock RNAi control (Figures 3, C and D). The extent of differentiation was quantified with secondary replating assays that revealed a three- to fivefold increase in differentiated colonies in comparison with the nontargeting control (Figure 3E). To assess the differentiation state of Zic3

knockdown cells, we assayed for changes in expression of key pluripotency genes (Figure 3A). Though the mouse ES cells showed clear morphological changes (Figure 3, C and D), surprisingly, there were only modest decreases (15–25%) in the expression of the key pluripotency genes Oct4 and Sox2 (Figure 3A), whereas Nanog expression decreased 40% relative to the nontargeting control. We performed the same experiment with human ES cells (HuES9). Although there was 70% decrease in Zic3 transcript levels, Oct4 and Sox2 transcript levels remained unchanged and Nanog levels decreased by 25% (Figure 3A). These results indicate that Zic3 plays a role in maintaining ES cell pluripotency and its action is downstream of the dominant pluripotency factors Oct4, Sox2, and Nanog.

It is interesting that targeted repression of Zic3 induced morphological differentiation of ES cells while maintaining the expression of pluripotency marker genes in the transient knockdown experiments. We were interested in assessing the role of Zic3 in the maintenance of pluripotency. To determine the differentiation status of these cells we assayed by Q-RT-PCR for expression of markers that represent lineage-specific ES cell differentiation (Table 1). Zic3 knockdown in mouse and human ES cells resulted in an up-regulation of a panel of endodermal markers: Sox17 (3.5-fold), PDGFRA (3.2- to 5.5-fold in mouse ES cells; 2.7-fold in human ES cells), and Gata6 (2.5- to 3.5-fold; Figure 4). In addition, two more endodermal lineage genes Gata4 and Foxa2 were up-regulated in the E14 RNAi cells (2.5-fold). We also assayed the expression of mesendodermal, mesodermal, ectodermal, trophectodermal and Wnt-pathway markers in Zic3 RNAi cells. These markers remained unchanged relative to the nontargeting control in both mouse and human RNAi experiments (Figure 4). These results indicate that Zic3 could play a specific role in maintaining ES cell pluripotency by suppressing endodermal specification.

Rescue of RNAi-induced Zic3 Phenotype

Our RNAi experiments have established a link between the expression of Zic3 and suppression of endodermal lineage specification. We observed consistent results using multiple siRNAs and shRNAs in both mouse and human ES cells. However, there is still concern that ES cell differentiation and marker gene expression were due to off-target effects of the RNAi. To address this concern we designed a Zic3 expression construct that was immune to RNAi and tested whether this construct could rescue the knockdown phenotypes.

The Zic3 RNAi-immune expression construct was engineered with five silent mutations in protein coding domain sequence (Supplementary Figure 1). As such, this construct (mutZic3) produces functional Zic3 protein, but with the added feature that it is resistant to RNAi targeting and

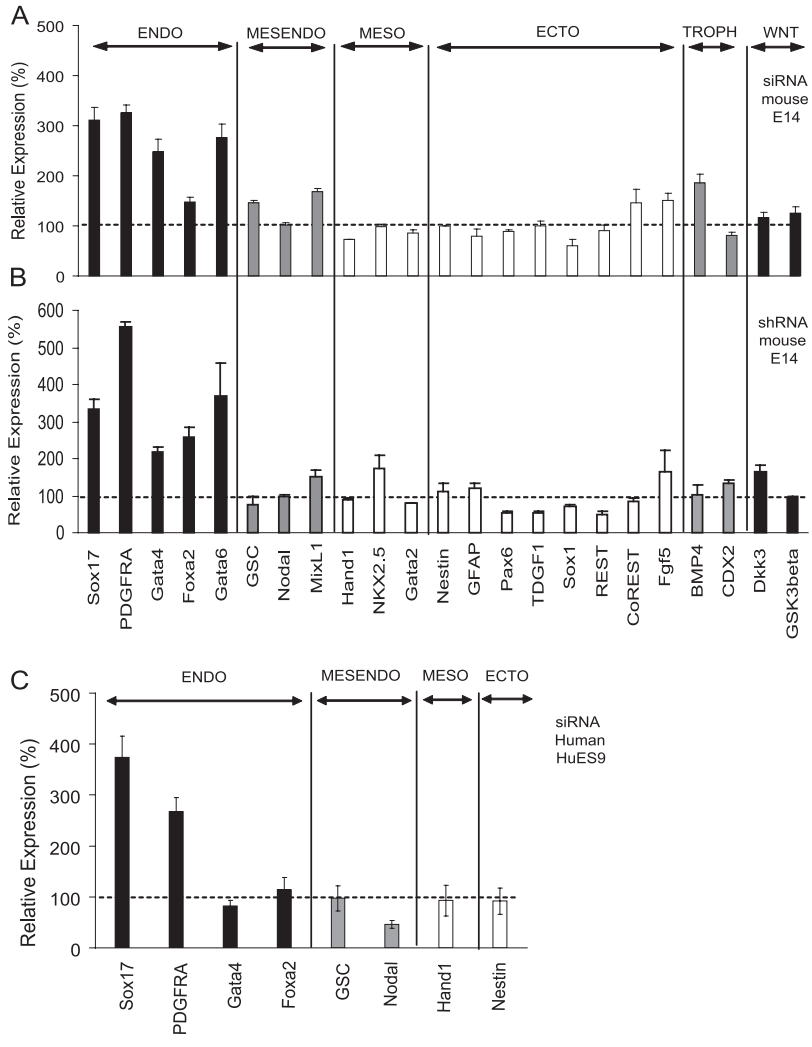

Figure 4. Effect of Zic3 RNAi on lineage marker gene expression. The panel of genes above was selected for their lineage specificity. Transcript levels of genes from the endodermal (ENDO), mesendodermal (MESENDO), mesdodermal (MESO), ectodermal (ECTO), trophectordermal (TROPH), and Wnt pathways in mouse and human ES cells were assayed by real-time PCR after Zic3 depletion by RNAi. (A) siRNA in mouse E14 cells. (B) shRNA in mouse E14 cells. (C) siRNA in human HuES9 cells. Mean levels ± SE are expressed as percentages relative to the nontargeting control (100%). The assays were read in duplicate and results were normalized to β-actin.

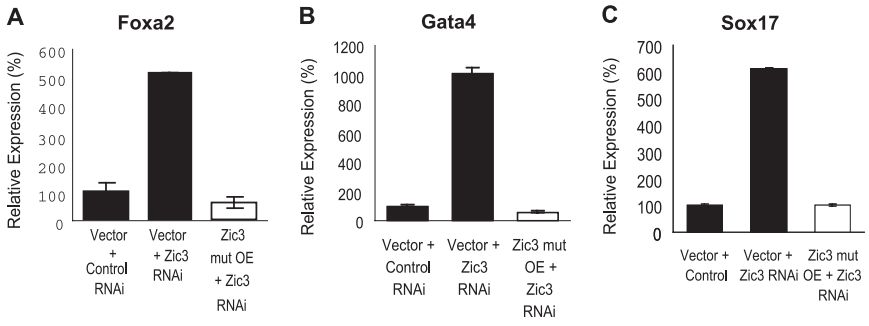

Figure 5. Zic3-immune construct specifically reverses changes in lineage marker expression levels caused by Zic3 RNAi. (A–C) Zic3 rescue experiments demonstrating the specificity of Zic3 RNAi and reversibility of lineage marker expression. E14 cells cotransfected with the Zic3 RNAi-immune overexpression construct and Zic3 RNAi vector demonstrated notable suppression of endodermal markers Foxa2, Gata4, and Sox17, relative to Zic3 RNAi cotransfected with the empty vector control. Zic3 immune real-time PCR analysis was conducted 3 d after transfection. β-Actin was used as an internal control for normalization. The measurements were done in duplicates and the average of the normalized ratio of target gene/β-actin was calculated and presented with SD. Relative expressions calculated with respect to the control experiment (Vector + control RNAi) at 100%. Transfection schemes are represented in Supplementary Table 1b.

degradation. Using this mutZic3 construct, we determined the specificity of the endodermal lineage specification produced by Zic3 knockdown. First, the expression levels of endodermal markers Foxa2, Gata4, and Sox17 were induced in ES cells cotransfected with empty vector and Zic3-RNAi, compared with cells cotransfeceted with empty vector and GFP-RNAi (6.5-, 10.1-, and 8.7-fold, for Foxa2, Gata4, and Sox17, respectively, Figure 5, A–C). However, ES cells that express the mutZic3 (RNAi immune construct) showed no induction of endodermal markers by Zic3-RNAi. (Figure 5, A–C). These experiments indicate that our RNAi results are not due to off-target effects and further support our conclusions that Zic3 plays a role in maintaining the pluripotency of ES cells.

Effects of Simultaneous Reduction of Zic2 and Zic3 Expression

Zic2 is another member of the Zic-family of transcription factors. Zic2 is expressed in ES cells and its expression is down-regulated upon differentiation (Brandenberger *et al.*, 2004; Wei *et al.*, 2005). Zic2 may also be regulated by Oct4, Sox2, and Nanog as binding sites for these TFs have been mapped to the Zic2 gene by chromatin immunoprecipitation (ChIP; Supplementary Figure 2). It was interesting that Zic3 RNAi resulted in a twofold increase in Zic2 (Figure 6A), and this raised the possibility that Zic2 may be compensating for the reduction in Zic3 levels. Knockdown of Zic2 expression by siRNA (75% reduction in RNA levels) did not produce any effect on lineage marker expression (Figure 6B). To determine if Zic2 compensated for the absence of Zic3, we performed a double RNAi experiment with Zic2 and Zic3 in ES cells. The double knockdown prevented Zic2 levels from increasing in a compensatory manner as observed in the Zic3 single knockdown (Figure 6C). Interestingly, endodermal specification was markedly enhanced after the Zic2 and Zic3 double knockdown as demonstrated by increased expression of Sox17 (4.7-fold), PDGFRA (8.7-fold), and Gata4 (3.1-fold), which is more robust than observed for all three markers (Sox17, 3.1-fold; PDGFRA, 3.3-fold; Gata4, 1.5-fold) when Zic3 alone was reduced (Figure 6D). Thus, we demonstrate that in the absence of Zic3, Zic2 is able to compensate at least partially to reduce the extent of endodermal specification in ES cells.

Zic3 Clonal Knockdown Lines Show Enhanced Endodermal Specification

To determine if endodermal markers were up-regulated in the same cells in which Zic3 was depleted, three clonal lines were generated that stably expressed Zic3 shRNA. As anticipated, Zic3 expression in the clonal lines was down-regulated 60% relative to vector-only control lines (Figure 7A). This knockdown is slightly less robust than in the transient Zic3 knockdowns where depletion of Zic3 expression by 70–80% was observed (Figure 3A). The pluripotency genes Oct4 and Sox2 were reduced between 20 and 30% relative to controls in all three clonal knockdown lines, whereas Nanog was reduced by 80% (Figure 7A). The endodermal genes PDGFRA, Gata4, Gata6, and Sox7 were 30-fold higher than in the controls, whereas Sox17 was up-regulated between 60- to 80-fold and FoxA2 was increased by 80- to 120-fold in all three Zic3 knockdown lines (Figure 7B). The induction of endodermal markers here was substantially greater than observed in the transient Zic3 knockdowns. Markers of the mesendoderm, mesoderm, ectoderm, trophectoderm, and Wnt pathways remained essentially unchanged (<2-fold) in the Zic3 knockdown lines (Figure 7C). Thus the specific up-regulation of endodermal gene expression in the clonal lines is consistent, in fact more pronounced, with our observations in the transient knockdowns (Figure 4).

To ascertain if there were corresponding increases in endodermal protein levels, immunocytochemistry was performed against FoxA2, Gata6, and PDGFRA in the clonal lines. The Zic3 knockdown lines consistently demonstrated robust endodermal marker staining (Figure 8A) that was absent in the vector control lines (Supplementary Figure 3). Oct4 staining was also observed in the cells that were positive for endodermal marker expression (Figure 8A). Interestingly, although the Zic3 clonal knockdown lines expressed Oct4 and SSEA-1 (Figure 8B), Nanog protein expression was significantly reduced relative to the vector control lines (Figure 8C). This agreed with the down-regulation observed in Nanog gene expression levels in the Zic3 knockdown lines (Figure 7A) and raises the possibility that Nanog gene expression is regulated by Zic3.

DISCUSSION

The work presented here demonstrates that Zic3 plays a key regulatory role in controlling ES cell differentiation. In this article, we have demonstrated that the expression pattern of Zic3 in ES cells corresponds closely with that of known regulators of pluripotency Oct4, Nanog, Sox2, which have high levels of expression in the undifferentiated state and decrease rapidly upon differentiation (Figure 1). Our findings in mouse ES cells are consistent with results from human ES cells (Brandenberger *et al.*, 2004). The differences we observed in Zic3 expression levels between pluripotent and early differentiation phases imply a potentially significant role for Zic3 in ES cell pluripotency. In addition, ChIP mapping by us and others has revealed Oct4, Nanog, and

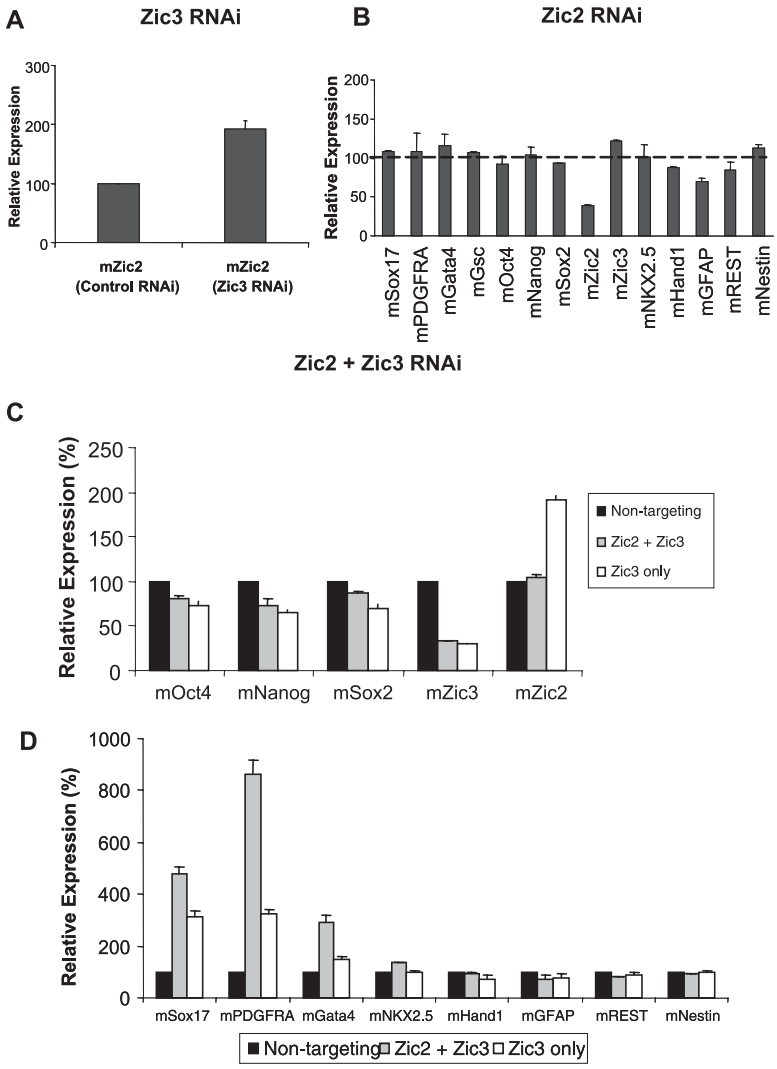

Figure 6. Effect of Zic2 and Zic3 double knockdown. The genes were assayed by real-time PCR in triplicate and normalized to a β-actin control. Mean levels $\pm$ SE are expressed as percentages relative to the nontargeting control. (A) Zic2 gene expression increased twofold with Zic3 transient knockdown 4 d after transfection. (B) Zic2 knockdown by siRNA was specific but did not produce changes in lineage markers assayed. (C) Zic2 and Zic3 coknockdown produced specific knockdown of Zic3 and at the same time prevented compensatory increase of Zic2 expression in ES cells. (D) The expression of endodermal lineage markers Sox17, PDGFRA, and Gata4 showed a similar pattern of up-regulation as in the Zic3 single knockdown, but was significantly enhanced in this Zic2/Zic3 coknockdown.

Sox2 co-occupancy on the Zic3 regulatory region, suggesting that Zic3 may be coordinately regulated by Oct4, Nanog, and Sox2 in mouse and human ES cells (Boyer *et al.,* 2005; Loh *et al.,* 2006; Figure 1C). These observations together led to our hypothesis that Zic3 functions to maintain the pluripotent state of ES cells. Here we characterized the relationship of Zic3 with that of the key stem cell regulatory factors and uncovered a role for Zic3 in the maintenance of ES cell pluripotency.

Our first objective was to assess the nature of interactions between Oct4, Nanog, and Sox2 with the Zic3 regulatory region. In constructing the transcriptional network around the key pluripotency genes, it is important to establish the outcome of transcription factor binding on downstream genes. We addressed this using a combinatorial approach encompassing the results of ChIP mapping and RNAi, demonstrating that ablation of Oct4, Nanog, and Sox2 in mouse ES cells resulted in a significant decrease in Zic3 expression (Figure 3A). Because Zic3 has already been implicated as a target of Oct4, Nanog, and Sox2 in ChIP experiments (Boyer *et al.,* 2005; Loh *et al.,* 2006), the concern of nondirect or secondary effects of RNAi was significantly reduced (Blais and Dynlacht, 2005). We thus concluded that the interaction of Oct4, Nanog, and Sox2 with the regulatory region of the Zic3 gene serves to enhance target gene expression. In other words, the key pluripotency regulators function as transcriptional activators of Zic3 in ES cells (Figure 9). This point is underscored by our results with Nanog overexpression and binding site mutagenesis assays, which demonstrate a positive association between Nanog binding and Zic3 expression. We thus demonstrate positive functional interactions between the key pluripotency regulators and the Zic3 gene regulatory region.

Because transcriptional networks are also known to feature autoregulatory loops (Lee *et al.,* 2002; Blais and Dynlacht, 2005), we also asked if the inverse relationship was true, that is, whether Zic3 regulates expression of the key regulatory genes. We observed that Oct4 and Sox2 levels remained largely unperturbed by the ablation of Zic3 expression (Figures 3A and 7A). In the absence of clear changes despite a robust Zic3 knockdown, our data places Zic3 downstream of Oct4 and Sox2 in the ES cell transcriptional networks as illustrated in Figure 9. In addition, we found that Nanog expression decreased significantly in the Zic3 clonal knockdown lines (Figures 7A and 8C). It remains to be determined whether Zic3 directly regulates the expression of Nanog in embryonic stem cells.

ES cells are derived from the inner cell mass of the blastocyst and, as such, are able to undergo unlimited self-renewal and differentiation into the three germ layers of the

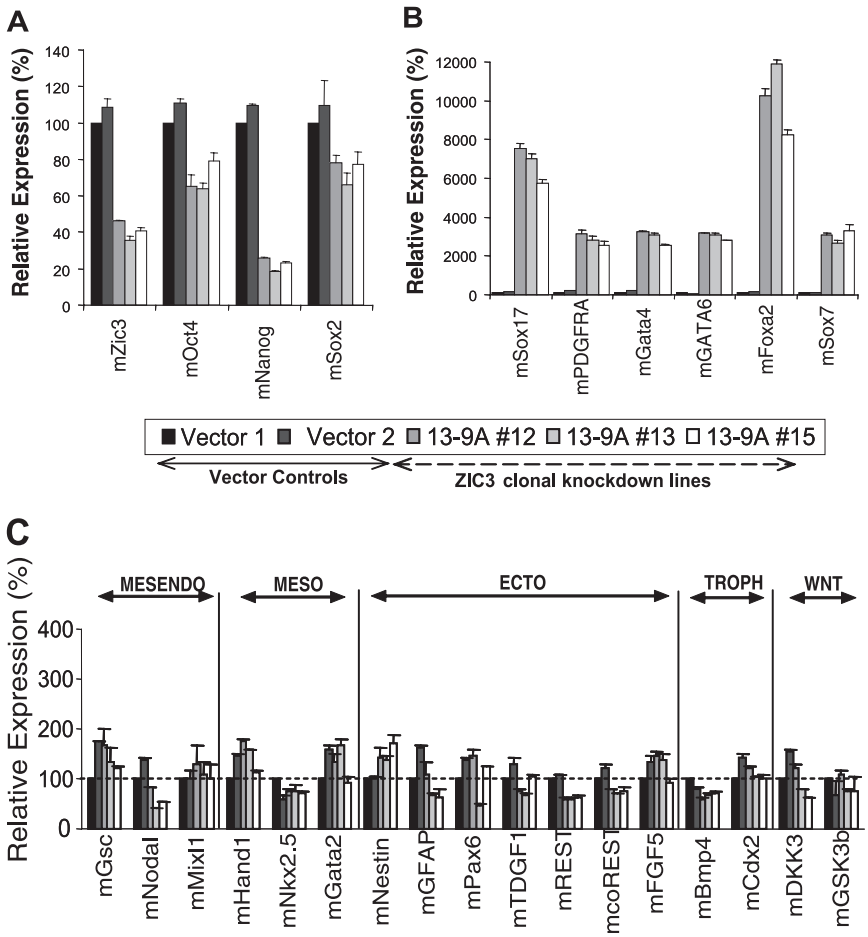

Figure 7. Zic3 knockdown clonal lines demonstrate endodermal gene marker specification. Three Zic3 knockdown clonal lines and two vector controls were assayed as indicated in the diagrams. (A) The pluripotency markers Oct4 and Sox2 were slightly down-regulated between 20 and 30%, whereas Zic3 and Nanog decreased significantly between 60 and 80% relative to the vector controls. (B) All endodermal markers assayed in the knockdown lines were significantly up-regulated between 20- and 120-fold relative to the control lines. (C) Mesendodermal, mesodermal, ectodermal, trophectodermal, and Wnt pathway genes did not change significantly in knockdown lines, demonstrating <2-fold changes relative to the vector controls. Gene expression levels were assayed by real-time PCR. The samples were assayed in triplicate and normalized to endogenous β-actin. Mean values $\pm$ SE are plotted as percentages relative to the vector control.

embryo: mesoderm, ectoderm, and endoderm (Evans and Kaufman, 1981; Martin, 1981). In the pluripotent state, ES cells remain undifferentiated and do not express specific lineage markers. We were interested in examining the effect of Zic3 knockdown on the maintenance of ES pluripotency using specific lineage markers as an assessment of differentiation after Zic3 knockdown (Table 1). Here we show that ablation of Zic3 expression in both mouse and human ES cells resulted in a significant increase in markers of endodermal lineage (Figures 4, 7, and 8). These results suggest that Zic3 may have an important role in preventing endodermal specification in ES cells.

Many reports support this observation: First, Zic3 knockdown in ES cells induced expression of Gata4 and Gata6, and forced expression of Gata4 and Gata 6 in ES cells result in differentiation toward extraembryonic endoderm (Fujikura *et al.*, 2002). Further strengthening this association is the fact that all other endodermal markers assayed (PDGFRA, Sox17, and FoxA2) are also expressed in extraembryonic endoderm derivatives (Kunath *et al.*, 2005). Second, Zic3 regulates Nodal expression through direct interaction with its promoter during gastrulation, and it has been shown that Nodal expression is essential in proper specification of the embryonic visceral endoderm (Mesnard *et al.*, 2006). This significance is underscored by studies reporting that the earliest abnormalities observed in Zic3 null mice are defects in proper patterning of the anterior visceral endoderm (Ware *et al.*, 2006b). Finally, Zic3 clonal knockdown lines exhibit a significant decrease in Nanog gene expression (Figures 7A and 8C), and several groups have reported that RNAi-mediated depletion of Nanog expression resulted in an induction of extraembryonic endoderm markers Gata4 and Gata6 (Mitsui *et al.*, 2003; Hyslop *et al.*, 2005; Hough *et al.*, 2006).

Here we have shown that Zic3 functions as a gatekeeper of pluripotency in ES cells by preventing their differentiation into cells that express endodermal markers. Corroborating this, we have found that Nanog expression is significantly reduced in the Zic3 clonal lines. This reduction is noteworthy as Nanog is a key regulator of pluripotency in ES cells (Chambers *et al.*, 2003), and it is well established that disruption of Nanog expression results in development of extraembryonic endoderm character in ES cells (Mitsui *et al.*, 2003; Hyslop *et al.*, 2005; Hough *et al.*, 2006). Thus, we demonstrate here an important role for Zic3 in the maintenance of pluripotency in ES cells through prevention of endodermal lineage specification, and we suggest that its action may in part be mediated through the key pluripotency regulator Nanog (Figure 9).

The role of Zic3 in preventing endodermal specification is further supported by evidence indicating its restricted expression within the mesoderm and ectoderm lineages during gastrulation (Herman and El-Hodiri, 2002). In addition, Zic3 activity has been specifically implicated in the mesodermal and ectodermal molecular pathways in the early developing embryo (Nakata *et al.*, 1997; Kitaguchi *et al.*, 2002; Maeda *et al.*, 2002; Kelly *et al.*, 2006). These data in combination with our results suggest that although Zic3 is instructive for mesodermal and ectodermal specification in embryonic development, it may simultaneously function as a repressor of ectopic endodermal induction in these tissues.

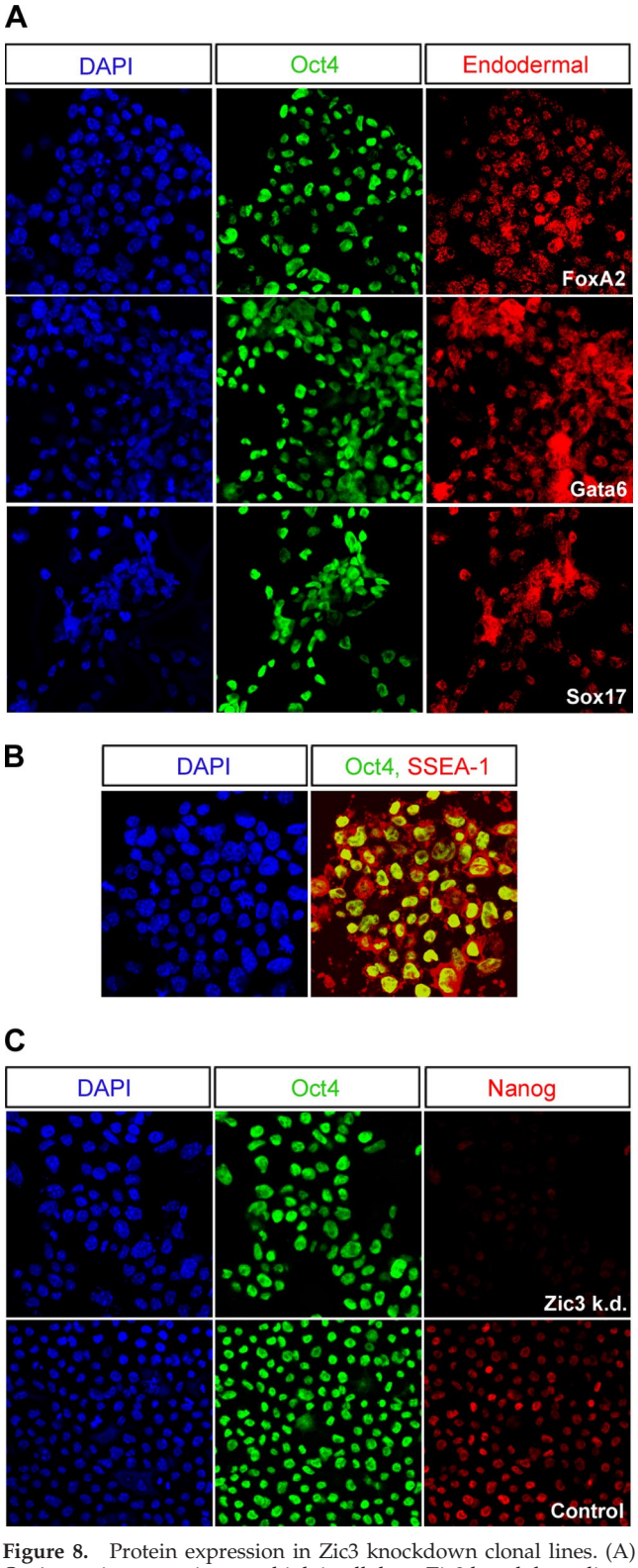

Figure 8. Protein expression in Zic3 knockdown clonal lines. (A) Oct4 protein expression was high in all three Zic3 knockdown lines, and the expression of specific endodermal marker proteins Foxa2, Gata6, and Sox17 was observed in the same cells. (B) The Zic3 knockdown lines expressed stem cell surface protein, SSEA-1, which is specific to murine ES cells. (C) The Zic3 clonal knockdown lines demonstrated a significant decrease in Nanog expression.

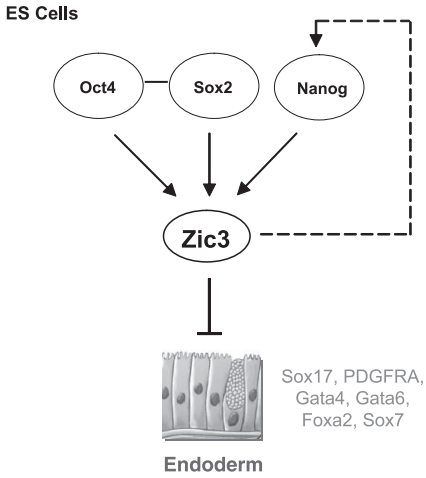

Figure 9. A model of Zic3 function in embryonic stem cells. Zic3 contributes to the maintenance of pluripotency by operating downstream of Oct4, Nanog, and Sox2 to inhibit endoderm lineage specification as characterized by endodermal markers Sox17, PDGFRA, Gata4, Gata6, Foxa2, and Sox7. The presence of Zic3 also maintains the expression of the homeodomain protein Nanog, a key regulator of pluripotency in embryonic stem cells.

The transcription factor Zic3 shares five highly conserved Zinc finger domains with family members Zic1, Zic2, Zic4, and Zic5 (Aruga *et al.*, 1994, 1996a,b, 2004). Their partially overlapping spatial and temporal patterns of expression during early development suggests potential functional redundancy between the Zic family members (Nagai *et al.*, 1997; Elms *et al.*, 2004). We observed that Zic2 gene levels were up-regulated when Zic3 expression was reduced (Figure 6A). Because Zic2 is also differentially expressed between pluripotent and differentiation states of ES cells (Brandenberger *et al.*, 2004; Wei *et al.*, 2005) and binding of the key pluripotency transcription factor Nanog has been mapped to the Zic2 regulatory region (Supplementary Figure 2), we reasoned that Zic2 may participate in the regulation of ES cell pluripotency along with Zic3. To unveil the possible effects of functional redundancy between Zic2 and Zic3, a double knockdown was performed in mouse ES cells. We report that repression of Zic2 and Zic3 expression significantly enhanced endoderm specification in ES cells (Figure 6C). The evidence that Nanog binds to the Zic2 regulatory region suggests that it may be involved in similar pathways as Zic3 in repressing endoderm expression. Thus, Zic2 and Zic3 may participate in redundant or partially overlapping networks to silence endoderm specifying gene expression and contribute to the maintenance of pluripotency in ES cells.

CONCLUSION

In this article, we expand on the significance of Zic3 as a target of the key stem cell regulatory factors in ES cells. Our results highlight a role for Zic3 in the maintenance of pluripotency downstream of Oct4 and Sox2, and uncovers its role as a gatekeeper controlling differentiation of ES cells into endoderm-specific lineages. In support of this, we present evidence that a key regulator of pluripotency, Nanog, which is shown to be important in repressing endodermal lineage specification, may directly or indirectly be regulated by Zic3 in ES cells. Having now established that Zic3 plays an important role in maintenance of pluripotency,

it will be valuable to search for Zic3-regulated target genes, which will extend our understanding of the transcriptional network that governs lineage specification. The elucidation of molecular signatures of early ES cells in this manner will contribute to validation and extension of the ES cell transcriptional network beyond Oct4, Nanog, and Sox2. The critical need to dissect their transcriptional networks is underscored by their potential to yield critical insights into genetic mechanisms at the earliest stages of embryo development and to provide significant inroads into the properties ES cell unlimited growth and differentiation potential that will render them therapeutically useful.

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