Zic3 Is Required for Maintenance of Pluripotency in Embryonic Stem Cells

Linda Shushan Lim,*† Yuin-Han Loh,‡† Weiwei Zhang,‡§ Yixun Li,‖ Xi Chen,‡§ Yinan Wang,‡§ Manjiri Bakre,* Huck-Hui Ng,‡§ and Lawrence W. Stanton*§

*Stem Cell and Developmental Biology Group, †Gene Regulation Laboratory, and ‡Information and Mathematical Sciences Group, Genome Institute of Singapore, Singapore 138672; and §Department of Biological Sciences, National University of Singapore, Singapore 117543

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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 C2H2 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., 2005).
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., 2002; Kelly et al., 2006).

Zic3 mutations are associated with X-linked heterotaxy, a disorder characterized by disruptions in embryonic lateralization and midline developmental field defect (Gebbia et al., 1997). In Zic3 mutant organisms *situs ambiguous* 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 organs, mirror-image inversions, and left-right isomerism (Ware 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 interact with the Zic3 regulatory 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

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Description</th>
<th>Lineage</th>
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<tbody>
<tr>
<td>Sox17</td>
<td>SRY-box containing gene 17</td>
<td>Endoderm</td>
</tr>
<tr>
<td>PDGFRA</td>
<td>Platelet-derived growth factor receptor, alpha</td>
<td>Endoderm</td>
</tr>
<tr>
<td>Gata4</td>
<td>GATA binding protein 4</td>
<td>Endoderm</td>
</tr>
<tr>
<td>Gata6</td>
<td>GATA binding protein 6</td>
<td>Endoderm</td>
</tr>
<tr>
<td>Foxa2</td>
<td>Forkhead box A2</td>
<td>Endoderm</td>
</tr>
<tr>
<td>GSC</td>
<td>Goosecoid</td>
<td>Mesendoderm</td>
</tr>
<tr>
<td>Nodal</td>
<td>Nodal</td>
<td>Mesendoderm</td>
</tr>
<tr>
<td>MixL1</td>
<td>Mix1 homebox-like 1</td>
<td>Mesendoderm</td>
</tr>
<tr>
<td>Hand1</td>
<td>Heart and neural crest derivatives expressed 1</td>
<td>Mesoderm</td>
</tr>
<tr>
<td>Nkx2.5</td>
<td>NK2 transcription factor related, locus 5</td>
<td>Mesoderm</td>
</tr>
<tr>
<td>Gata2</td>
<td>GATA binding protein 2</td>
<td>Mesoderm</td>
</tr>
<tr>
<td>Nestin</td>
<td>Nestin</td>
<td>Ectoderm</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
<td>Ectoderm</td>
</tr>
<tr>
<td>Pax6</td>
<td>Paired box gene 6</td>
<td>Ectoderm</td>
</tr>
<tr>
<td>TDGF1</td>
<td>Teratocarcinoma-derived growth factor/Cripto</td>
<td>Ectoderm</td>
</tr>
<tr>
<td>Sox1</td>
<td>SRY-box containing gene 1</td>
<td>Ectoderm</td>
</tr>
<tr>
<td>REST</td>
<td>RE1-silencing transcription factor</td>
<td>Ectoderm</td>
</tr>
<tr>
<td>CoREST</td>
<td>REST Co-repressor 1</td>
<td>Ectoderm</td>
</tr>
<tr>
<td>FGF5</td>
<td>Fibroblast growth factor 5</td>
<td>Ectoderm</td>
</tr>
<tr>
<td>BMP4</td>
<td>Bone morphogenetic protein 4</td>
<td>Trophoderm</td>
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<tr>
<td>CDX2</td>
<td>Caudal type homeobox 2</td>
<td>Trophoderm</td>
</tr>
<tr>
<td>DKK3</td>
<td>Dickkopf homolog 3</td>
<td>Wnt pathway</td>
</tr>
<tr>
<td>Gsk3beta</td>
<td>Glycogen synthase kinase 3 beta</td>
<td>Wnt pathway</td>
</tr>
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</table>
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-Leukemia 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, filtered 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 siCONTROL 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 × 10⁵ cells per well. Retransfections were performed on pre-adherent 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 × 10⁵ 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’-GAAAGGCTGTGACANAAGGCTGTGAC-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 × 10⁵ 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-replacing Assay

ES cells were transfected with Zic3- or empty pSUPER shRNA constructs and selected 24 h later with puromycin at 3.0 μg/ml over 4 d. At the end of 4 d 4-5 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 × 10⁴ 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 50 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 (20X 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.
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 immuno-chemistry, cells were seeded at a density of 1.0 × 10⁶ 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% 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'-TAATAacgcgtTTAGAGGTCAAACCAT-3' and reverse, 5'-TATATagatCTAGTAGTCAAACTGGATT-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 × 10⁶ 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 was 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 assessed 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
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., 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.

**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 was observed with Zic3 RNAi relative to mock-transfected control.

**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 was observed with Zic3 RNAi relative to mock-transfected control.
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, trophodermal 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
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 cotransfected 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 demonstrated 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, trophoderm, 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, Gata4, 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
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 pluripo-
tent state of ES cells. Here we characterized the relation-
ship 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
encapsulating the results of ChIP mapping and RNAi, dem-
onstrating that ablation of Oct4, Nanog, and Sox2 in mouse
ES cells resulted in a significant decrease in Zic3 expres-
sion (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 transcrip-
tional 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 ex-
pression. We thus demonstrate positive functional interac-
tions between the key pluripotency regulators and the Zic3
gene regulatory region.

Because transcriptional networks are also known to fea-
ture 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 ex-
pression (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 transcrip-
tional 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 expres-
sion of Nanog in embryonic stem cells.

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

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 ± SE
are expressed as percentages relative to the nontarget-
ing 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 expres-
sion 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.
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 Gata6 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.
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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