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Regulation of ES Cell Self Renewal and Pluripotency by Foxd3

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Abstract

The Foxd3 forkhead transcription factor is required for maintaining pluripotent cells in the early mouse embryo and for the establishment of murine embryonic stem (ES) cell lines. To begin to understand the role of Foxd3 in ES cell maintenance, we derived ES cell lines from blastocysts that carried two conditional Foxd3 alleles and a tamoxifen-inducible Cre transgene. Tamoxifen treatment produced a rapid and near complete loss of Foxd3 mRNA and protein. *Foxd3*-deficient ES cells maintained a normal proliferation rate but displayed increased apoptosis, and clonally dispersed ES cells showed a decreased ability to self-renew. Under either self-renewal or differentiation-promoting culture conditions we observed a strong, precocious differentiation of *Foxd3* mutant ES cells along multiple lineages including trophoblast, endoderm and mesoderm. This profound alteration in biological behavior occurred in the face of continued expression of factors known to induce pluripotency including Oct4, Sox2 and Nanog. We present a model for the role of Foxd3 in repressing differentiation, promoting self-renewal and maintaining survival of mouse ES cells.

Keywords

Foxd3; ES cell; self-renewal; transcription factor

INTRODUCTION

Murine embryonic stem cells (ES cells) are derived from the inner cell mass (ICM) of blastocyst stage embryos and can be maintained indefinitely *in vitro* while retaining the ability to subsequently differentiate into all the cells of the adult animal. Understanding properties of ES cells and how self-renewal and pluripotency are regulated will have a large impact on developmental biology studies and regenerative medicine. Several transcription factors are required for ES cell self-renewal and pluripotency, including Oct4, Sox2 and Nanog, and inactivation of these genes leads to loss of pluripotent stem cells and aberrant differentiation into extraembryonic trophoblast in the case of Oct4 and Sox2, or primitive endoderm in the case of Nanog¹⁻⁵. Recently, overexpression of a cocktail of transcription factors (Oct4, Sox2, c-Myc and Klf4 or Oct4, Sox2, Lin-28 and Nanog) has resulted in the induction of pluripotency in somatic cells⁶⁻¹⁰. These “induced pluripotent stem cells” (iPSCs) have all the properties of ES cells, but the mechanism of this induction is still unclear. Identification of factors immediately downstream of these transcription factors will be crucial.

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Foxd3 is a forkhead transcription factor required for maintenance of progenitor cells in the ICM, trophoblast and neural crest lineages¹¹⁻¹³. *Foxd3*^{-/-} embryos die shortly after implantation and cells in the mutant ICM and epiblast undergo extensive programmed cell death¹¹. ES cells express *Foxd3* and expression is dramatically downregulated when cells are induced to differentiate¹⁴, suggesting that Foxd3 expression in pluripotent stem cells is functionally significant. Together, this work illustrates the important role Foxd3 plays to maintain multipotent progenitor cells from divergent embryonic lineages, but the early lethality of *Foxd3*^{-/-} embryos and therefore inability to establish *Foxd3*^{-/-} ES cell lines, hampered efforts to study the role Foxd3 plays in ES cell maintenance.

To circumvent this problem, we derived ES cell lines in which Cre-mediated inactivation of Foxd3 function can be temporally regulated. These *Foxd3*^{fl/fl};CAGG *Cre-ER*TM (*Foxd3*^{fl/fl}; *Cre-ER*) ES cells are indistinguishable from normal ES cells in culture, and the *Foxd3* coding region is deleted when cells are cultured in the presence of 4-hydroxytamoxifen (TM). Using this inducible system, we demonstrate that Foxd3 is not required for cell proliferation, but that mutant ES cells undergo increased apoptosis indicating Foxd3 is required for ES cell survival. Mutant ES cells were defective in their ability to form colonies from single cells, illustrating a requirement for Foxd3 in stem cell self-renewal. At the same time, while maintained under differentiation inhibiting conditions, *Foxd3* mutant ES cells do not respond to these cues and undergo extensive differentiation despite the maintenance of expression of multiple stem cell genes. Together, our results shape a deeper understanding of the biological roles of this transcription factor in murine ES cells and allow us to propose a model that will further our comprehension of mechanisms regulating maintenance of self-renewal and multipotency, the defining characteristics of all stem cells.

MATERIALS AND METHODS

Generation of Inducible *Foxd3* Mutant mES Cell Lines

Foxd3^{fl} mice were maintained on a 129S6/SvEvTac (Taconic) genetic background¹³. Mice carrying a tamoxifen-inducible variant of Cre recombinase (*CAGG Cre-ER*TM, also called Tg (cre/Esr1)5Amc)¹⁵ (The Jackson Laboratory) were backcrossed four times to 129S6/SvEvTac mice. The resulting animals were crossed to *Foxd3*^{fl/fl} mice and a line of *Foxd3*^{fl/fl};CAGG *Cre-ER*TM established. These were interbred and blastocysts harvested at 3.5 dpc using standard methods^{16, 17}. Blastocysts were cultured on irradiated STO fibroblasts in ES cell medium supplemented with 50μM MEK1 inhibitor PD98059 (Cell Signaling Technology). After 3-4 days, ICM outgrowths were isolated, trypsinized in microdrops, and cell suspensions transferred to fresh feeder layers. After 4-5 days, ES cell lines were obvious in the cultures. Lines were cryopreserved at passage number 3-4 and samples were lysed for DNA extraction. Individual cell lines were genotyped for the *Foxd3*^{fl} allele and presence of *Cre* using PCR as described¹³. Animal care was in accordance with Vanderbilt University IACUC guidelines.

ES Cell Culture

ES cells were cultured on irradiated mouse embryonic fibroblast (MEF) feeder cells using standard protocols¹⁷. 4-hydroxytamoxifen (Sigma) was dissolved in ethanol at 1mM stock concentration, and added to the medium on a daily basis at the concentration of 2μM unless otherwise specified. Differentiation of ES cells was carried out by generating embryoid bodies (EBs) in suspension culture on ultra-low attachment dishes (Corning) in ES medium without addition of LIF.

Cell Number Analysis and Self-Renewal Assay

For cell number analysis, ES cells were plated at a density of 5×10^4 cells/well in 12-well dishes. Two to four days after plating, cells were dissociated with trypsin and trituration to a single

cell suspension and manually counted with a hemocytometer. Cell numbers were measured for multiple cell lines from at least three independent experiments.

To assay for self-renewal, ES cells treated with or without 2 μ M TM for two days were dissociated and plated at clonal density (10 cells/cm²) on MEF feeders in 12-well dishes. At day 4, cells were fixed and stained for alkaline phosphatase activity according to manufacturer's instruction (Millipore) and counter-stained with 4,6-diamidino-2-phenylindole (DAPI). Colony formation efficiency was calculated by the counting the number of AP-positive colonies divided by the total number of cells plated. Number of cells per colony was determined by manually counting DAPI-stained cells. Positively stained cells were manually counted by two individuals who were blinded to the genotypes and TM treatment. Colony formation efficiency and number of cells/colony were determined from three independent experiments.

Immunocytochemistry

ES cells were washed with phosphate-buffered saline (PBS), and fixed with 4% paraformaldehyde (PFA) in PBS for 30 minutes at room temperature. EBs were washed with PBS, and fixed with 4% PFA at 4°C for 1 hour. After sinking in 30% sucrose at 4°C overnight, EBs were embedded in OCT, and cryosectioned at 10 μ m. For immunofluorescence, cells or sections were permeabilized and blocked using 0.1% Triton X-100 (Sigma) and 1% goat serum (Jackson ImmunoResearch) in PBS. Primary antibodies were diluted in blocking buffer and incubated overnight at 4°C. Secondary antibodies diluted in blocking buffer were incubated at room temperature for 1 hour. Cells were counter-stained with DAPI. Images were captured using a Zeiss Observer A.1 fluorescent microscope and AxioCam MRc5 camera. Cells were counted manually by two individuals.

Primary antibodies used include: rabbit anti-Foxd3¹² (1:1000), rabbit anti-phospho-Histone 3 (pH3, 1:500; Millipore), mouse anti-Oct4 (1:500; Beckton Dickinson), rabbit anti-Sox2 (1:3000; kind gift from Dr. Larysa Pevny), rabbit anti-Nanog (1:500; Invitrogen), mouse anti-Foxa2 (1:50; Developmental Studies Hybridoma Bank), rabbit anti-Foxa2 (1:500; kind gift from Dr. Chin Chiang). Secondary antibodies used were Cy3 (Jackson ImmunoResearch) and Alexa 488 (Invitrogen) goat anti-rabbit or goat anti-mouse (1:500).

Proliferation and TUNEL Assays

500 μ g/ml of 5-bromo-2'-deoxyuridine (BrdU; Sigma) was added to the culture medium for one hour. Cells were then fixed with 4% PFA in PBS, washed with PBS, and treated with 2N HCl for 20 min. Immunocytochemistry was performed as described above using a mouse anti-BrdU (1:50; Beckton Dickinson) antibody. TUNEL assays were performed using the In Situ Cell Death Detection Kit (Roche) according to manufacturer's instructions. BrdU-positive, pH3-positive and TUNEL-positive cells were counted and the percentages calculated against total number of DAPI-stained cells. Data were collected from more than 3 independent experiments with more than 7000 DAPI-positive cells counted for each treatment.

RNA Isolation and RT-PCR

Total RNA was isolated using the RNeasy Mini Kit or RNeasy Micro Kit (Qiagen) followed by TURBO DNase treatment (Ambion) according to manufacturer's instructions. cDNA synthesis was carried out with 500ng of total RNA using SuperScript III qRT-PCR kit (Invitrogen). Real-time quantitative RT-PCR (qRT-PCR) was performed with ABI PRISM 7900 Sequence Detection System using SYBR Green Master Mix (ABI). Samples were run in duplicate and relative levels of each mRNA were examined by comparing Cycle Threshold (Ct) values for each reaction among samples using the *Hypoxanthine phosphoribosyl transferase (Hprt)* gene as reference. Relative expression levels of each mRNA were measured

from at least 3 independent experiments, and compared by Student's t-test. Primer sequences are listed in Supplemental Table 1.

RESULTS

Reduction of *Foxd3* Expression by 4-Hydroxytamoxifen Treatment

Foxd3 is required for maintenance of the ICM and the establishment of ES cell lines¹¹. Therefore, in order to generate a genetic lesion of *Foxd3* in ES cells, we needed to develop a regulatable system. The *Foxd3* conditional allele (*Foxd3^{fl/fl}*)¹³ was designed so that Cre-mediated recombination deletes the entire coding region of *Foxd3* gene. The *CAGG Cre-ERTM* transgenic line expresses a tamoxifen-regulated fusion protein of Cre fused to a modified ligand binding domain of the estrogen receptor (ER)¹⁵. *Foxd3^{fl/fl}; CAGG Cre-ERTM* (*Foxd3^{fl/fl}; Cre-ER*) mice appeared normal and healthy and were intercrossed to obtain blastocysts for ES cell derivation. We obtained experimental lines (*Foxd3^{fl/fl}; Cre-ER*) and control lines without the *Cre-ER* transgene (*Foxd3^{fl/fl}*) in equal numbers. Genotyping scheme is shown in Fig. 1A. PCR of genomic DNA isolated from wild type, *Foxd3^{fl/fl}; Cre-ER* and *Foxd3^{fl/fl}* ES cells confirmed the lack of wild type and presence of *Foxd3^{fl}* alleles in untreated *Foxd3^{fl/fl}; Cre-ER* and *Foxd3^{fl/fl}* cells, and the presence of the *Cre* transgene in *Foxd3^{fl/fl}; Cre-ER* cells (Fig. 1B). When cultured in complete ES cell medium without TM, ES cells of both genotypes appeared similar to wild type ES cells and displayed comparable growth rates. When TM was added to the culture medium, the presence and intensity of the PCR product from the *Foxd3^{fl}* allele was unchanged in *Foxd3^{fl/fl}* control ES cells. However, recombination of the *Foxd3^{fl}* allele was detected in TM-treated *Foxd3^{fl/fl}; Cre-ER* experimental cells (Fig. 1B). Cre-mediated recombination was not complete because some *Foxd3^{fl}* PCR product was detected in the TM treated cultures (Fig. 1B). Real-time quantitative PCR revealed that after 3 days of TM treatment, the PCR signal from the *Foxd3^{fl}* allele in TM-treated *Foxd3^{fl/fl}; Cre-ER* cultures ranged from 13 to 24% of levels in untreated ES cells (n=3 experiments, data not shown).

We analyzed four independent cell lines of each genotype for *Foxd3* expression at the mRNA and protein levels; E1, E2, E3 and E4 were experimental lines and C1, C2, C3, and C4 were control lines. Immunocytochemistry with a *Foxd3* antibody revealed that 70-90% of the ES cells in untreated cultures expressed *Foxd3* (Fig. 1E), consistent with the expression of *Foxd3* in the ICM¹¹. However, when cultured in the presence of TM, *Foxd3* mRNA and protein levels were significantly reduced in experimental cells but not control cells (Fig. 1C, D; see also Fig. 3A and C). Real time quantitative RT-PCR (qRT-PCR) analysis revealed a greater than 90% reduction of *Foxd3* mRNA in *Foxd3^{fl/fl}; Cre-ER* ES cells one day after TM treatment and after 2 days mRNA levels were nearly undetectable (Fig. 1D). Quantification of the number of *Foxd3* expressing cells by immunocytochemistry showed that after one day of TM treatment, about 15% of the cells still contained *Foxd3* protein (Fig. 1E, arrowheads). Two and three days of TM treatment resulted in further reduction of *Foxd3*-positive cells in the cultures (7.4% and 5.8% respectively) and a decrease in total *Foxd3* protein as measured by western blot analysis (data not shown), suggesting that Cre-mediated recombination occurred in the vast majority of cells under these conditions.

Foxd3 is Required for ES Cell Self-renewal and Survival

To examine whether *Foxd3* plays a role in growth and maintenance of ES cells, we quantified the effect of TM treatment on cell numbers in experimental and control ES cells. When *Foxd3^{fl/fl}; Cre-ER* ES cells were cultured in the presence of 2 μ M TM, the number of surviving cells after 2-4 days was significantly reduced compared to control cultures (Fig. 2A). Control *Foxd3^{fl/fl}* ES cells cultured in the absence or presence of 2 μ M TM had a similar number of surviving cells after 2-4 days of TM treatment (Fig. 2A). These results suggest that loss of *Foxd3*, but not treatment with TM, was responsible for the decreased cell numbers and colony

size (Supplemental Fig. 1A) in ES cells. A higher concentration of TM (4 μ M) showed a non-specific toxic effect, control cell lines cultured in the presence of 4 μ M TM had reduced number of cells (Fig. 2A). Therefore, we used 2 μ M TM for the rest of our experiments. Serial passaging of *Foxd3^{fl/fl}; Cre-ER* ES cells with TM treatment (ten passages) resulted in decreased number of surviving cells at each passage; however, cells did not die off and continued culture was possible in most cases (Supplemental Fig. 1B). However, many of these cells were *Foxd3*-positive, suggesting that they were selectively able to proliferate despite continued TM in the medium (data not shown).

Self-renewal of stem cells is defined by the ability of a single cell to give rise to a new colony of stem cells¹⁸⁻²⁰. Cells were cultured with or without TM for 2 days and plated at clonal density (10 cells/cm²). Alkaline phosphatase (AP) staining was performed after 4 days and immunocytochemistry confirmed that the mutant AP-positive colonies no longer expressed *Foxd3* (Fig. 2B). The efficiency of colony formation of untreated experimental cells was 44.4%, while TM-treated cells formed colonies at only 10.6% (Fig. 2C), suggesting a defect in self-renewal of *Foxd3*-deficient ES cells. In addition, although both untreated and 2 μ M TM-treated cultures of *Foxd3^{fl/fl}; Cre-ER* ES cell lines contained AP-positive colonies (Fig. 2B), treated colonies contained 3-4 fold fewer cells than control colonies (Fig. 2D). When the same assays were performed with *Foxd3^{fl/fl}* control ES cells, we saw no difference in either colony size or efficiency of colony formation with (42.7%) or without (45.6%) TM treatment (Supplemental Fig. 1C and D). These experiments demonstrate that *Foxd3* is required for self-renewal of ES cells.

The reduced cell numbers and colony size we observe in ES cell cultures could be caused by increased cell death and/or decreased cell proliferation. We measured programmed cell death by TUNEL labeling, and while control cultures without TM treatment exhibited few apoptotic cells, we observed more *Foxd3^{fl/fl}; Cre-ER* ES cells treated with TM undergoing apoptosis at 2 and 3 days after TM treatment compared to controls (Fig. 3A, arrows). Quantification of the percent of TUNEL-positive cells showed a two-fold increase in apoptosis after 2 or 3 days of TM treatment (Fig. 3B). There was no increase of apoptosis in *Foxd3^{fl/fl}* control ES cells treated with TM (Fig. 3A). These data indicate that *Foxd3* plays an important role in maintaining ES cell survival. In mutant colonies, *Foxd3* positive ES cells (Fig. 3A, arrowheads) and TUNEL positive cells (Fig. 3A, arrows) were observed, but *Foxd3*/TUNEL-double positive cells were rarely seen (2 out of 972 cells counted at day 2 and 3/1034 for day 3), suggesting a cell-autonomous function of *Foxd3* in preventing ES cells from programmed cell death. To examine proliferation of *Foxd3* mutant cells, we used BrdU incorporation to identify cells in S phase of the cell cycle, and phospho-Histone 3 (pH3) as a marker for cells in G2-M phase. In mutant cultures, many *Foxd3*-deficient ES cells were BrdU-positive (Fig. 3C, arrows), suggesting that *Foxd3* is not required for ES cells to enter S phase of the cell cycle. Quantification of BrdU-positive cells showed no significant difference between mutant and control cultures (Fig. 3D). Similarly, the percent of pH3-positive cells in *Foxd3* mutant ES cell cultures was similar to that of untreated cultures (Fig. 3E and F). Together, these data show that the deletion of *Foxd3* has no significant effect on ES cell proliferation, but results in increased cell death.

Maintenance of Stem Cell Gene Expression in *Foxd3* Mutant ES Cells

Pluripotent ES cells are maintained in an undifferentiated state by the function of a set of key transcription factors including Oct4, Sox2 and Nanog^{21, 22}, and overexpression of these transcription factors in combination with Klf4, c-Myc and/or Lin28 can reprogram somatic cells to adopt a stem cell phenotype^{7-10, 23}. To examine whether the loss of *Foxd3* affects the expression of these stem cell genes, we analyzed mRNA levels and protein expression of multiple stem cell transcription factors in *Foxd3* mutant ES cells. Immunocytochemistry for Oct4, Sox2 and Nanog showed that all three proteins were expressed in *Foxd3* mutant ES cells

after 3 days of TM treatment (Fig. 4A). Double immunocytochemistry for Oct4 and Foxd3 confirmed that most Oct4-positive cells in mutant cultures were Foxd3-deficient (Fig. 4A), suggesting that Foxd3 is not required for maintaining the expression of these stem cell genes. A time-course analysis of mRNA levels for these genes along with *Zfx*, *Esrrb*, *c-Myc*, *Tbx3* and *Klf4* by qRT-PCR revealed a small decrease of transcript levels of some of the genes after days 1 and 2 of TM treatment, and by day 3, expression levels were either unchanged or slightly increased (Fig. 4B). Oct4 protein levels in the treated experimental cultures were also maintained as measured by western blotting (data not shown). These findings indicate that Foxd3 is not required to maintain the expression of the stem cell genes analyzed, and suggest that Foxd3 may function downstream of these genes, or in a parallel pathway, in mouse ES cells.

Aberrant Differentiation of *Foxd3* Mutant ES Cells

When ES cells are cultured in the presence of mitotically inactivated embryonic fibroblast feeders, serum and LIF, the overwhelming majority of the cells maintain an undifferentiated phenotype. To examine the differentiation status of *Foxd3* mutant ES cells cultured under conditions that normally inhibit differentiation, we used qRT-PCR to monitor expression of differentiation markers for several lineages. A 2-4 fold increase in mRNA levels of primitive endoderm markers *Foxa2*, *AFP* and *Sox17* in TM treated cultures was observed (Fig. 5A), indicating increased differentiation to primitive endoderm of *Foxd3*-deficient ES cells. Trophectoderm is an extra-embryonic lineage that contributes to the placenta, and normally ES cells do not contribute to trophoctoderm in chimeras or *in vitro*²⁴. Despite this lineage restriction, *Foxd3* mutant ES cells showed a 12-, 3.4- and 4.4-fold increase in mRNAs for trophoctoderm markers *Cdx2*, *Fgfr2* and *Csh1/PLI*, respectively (Fig. 5B). After implantation, the ICM gives rise to primitive ectoderm/epiblast cells, which then contribute to cells in the three primary germ layers. One of the first differentiation markers expressed in epiblast cells *in vivo*, *Fgf5*²⁵, was increased 2-fold in *Foxd3* mutant ES cells (Fig. 5C). *Gooseoid* (*Gsc*) and *Brachyury* (*T*), molecular markers for mesendoderm²⁶, increased 5.4- and 12-fold, respectively (Fig. 5C). Together, these results suggest that Foxd3 normally functions to maintain ES cells in an undifferentiated state by repressing differentiation towards extra-embryonic and embryonic lineages. Interestingly, expression levels of neuroectoderm markers *Nestin*, *Pax6* and *Sox1* either decreased or remained the same in mutant cells (Fig. 5D), suggesting that Foxd3 selectively represses commitment of epiblast cells into mesoderm and endoderm lineages, but not ectoderm.

During lineage diversification ES cells up-regulate lineage-specific genes while down-regulating self-renewal genes. One possible mechanism for precocious and aberrant differentiation is the disruption of this coordinated regulation, with the induction of lineage specific gene networks at the same time that self-renewal gene expression is maintained. To test this possibility in *Foxd3* mutant ES cells, we performed double immunocytochemistry for the endoderm marker *Foxa2* and stem cell markers Oct4 and Sox2 (Fig. 5E). Both TM-treated *Foxd3*^{fl/fl}; *Cre-ER* cultures (Fig. 5E) and untreated cultures (data not shown) contained very few cells that were double-positive for *Foxa2* and Oct4 or Sox2 (Fig. 5E, arrowheads; 4-5% cells in both mutant and control cultures), suggesting the coordination between differentiation and self-renewal was not disrupted in *Foxd3* mutant ES cells. Surprisingly, the percent of Oct4-positive and *Foxa2*-positive cells in control and mutant cultures were similar (Fig. 5F). Because we observed increased apoptosis in Foxd3 mutant cultures, we performed TUNEL analysis and immunolocalization for either Oct4 or *Foxa2* to examine which population of cells preferentially undergoes apoptosis. Oct4 cells are mostly in the internal portion of the colonies while *Foxa2* cells are at the periphery (Fig 5E and G). In control cultures, TUNEL/Oct4 double-positive cells were rarely observed (Fig. 5H; 1.5% of total Oct4-positive cells); in mutant cultures, the percentage of TUNEL/Oct4 double-positive cells was similar (Fig. 5G and H;

2%). In contrast, we measured 6.6% TUNEL/Foxa2 double-positive cells in control cultures versus a significantly higher 28.7% TUNEL/Foxa2 double-positive cells in mutant cultures (Fig. 5G and H). Furthermore, in mutant cultures, not only did we observe TUNEL/Foxa2 double-positive cells that were DAPI-positive (Fig. 5G, arrowheads), we also observed many cells at the edge of colonies that were TUNEL/Foxa2 double-positive but DAPI-negative, suggesting these cells have lost their nuclear integrity (Fig. 5G, arrows). Together, these data indicate that *Foxd3* mutant ES cells undergo aberrant differentiation, and these differentiated cells have a higher tendency for apoptosis.

Precocious Differentiation of *Foxd3* Mutant Embryoid Bodies

ES cells not only have the capacity to self-renew *in vitro*, they are also capable of differentiating into multiple cell types. When ES cells are grown in suspension culture without LIF, the cells aggregate to form embryoid bodies (EBs). EBs differentiate in a manner similar to the early ICM²⁷⁻²⁹. First, cells in the outer layer of the EBs differentiate to primitive endoderm that forms a thick basement membrane separating the primitive endoderm layer from the inner core of the EBs²⁷. Next, EBs undergo cavitation similar to formation of the proamniotic cavity in the embryo³⁰. Cells in the inner core resemble epiblast/primitive ectoderm, and undergo differentiation into cells from all three germ layers.

Foxd3^{fl/fl}; *Cre-ER* ES cells formed EBs when cultured in the presence of 2μM TM. However, the EBs were much smaller than those from cells without TM treatment (Supplemental Fig. 2A), consistent with reduced cell survival in the absence of *Foxd3*. QRT-PCR confirmed that *Foxd3* mRNA was decreased in TM-treated cells in day 2 to day 8 EBs (Supplemental Fig. 2B and C). We examined primitive endoderm differentiation by qRT-PCR analysis of *Foxa2* mRNA levels in control and mutant EBs, and observed increased *Foxa2* expression in *Foxd3* mutant EBs at all stages examined (Fig. 6A). In control D4 EBs, *Foxa2* protein was detected primarily in the outer layer of primitive endoderm cells (Fig. 6B). In contrast, mutant EBs contained more *Foxa2*-positive cells, with many located within the core of the EBs. By D8, in addition to the outer layer *Foxa2*-positive primitive endoderm cells, a few *Foxa2*-positive cells were observed in the inner core of control EBs. The *Foxa2*-positive cells in *Foxd3* mutant EBs were more numerous and widespread in the inside of the EBs (Fig. 6B). Comparing the percent of *Foxa2*-positive cells in control and mutant EBs, we found a 5-fold and 3-fold increase in mutant EBs at D4 and D8, respectively (Fig. 6C). QRT-PCR analysis showed increased expression of another early endoderm marker, *Sox17*, in mutant EBs (Fig. 6D). mRNA levels of *Foxa2* and *Sox17* in mutant D2-D8 EBs were 3-fold to 18-fold higher than those in control EBs (Fig. 6A and 6D; note the logarithmic scale of these graphs). Two late endoderm markers *AFP* and *Alb1* showed a similar increase of expression in mutant EBs (Fig. 6E and F). Expression levels of the trophoblast marker *Cdx2*, and the mesendoderm marker *Gsc*, were also up-regulated in mutant EBs while the neurectoderm marker *Sox1* was slightly decreased (Fig. 6G - I). Together, these data show that *Foxd3* mutant EBs undergo spontaneous differentiation into the same repertoire of lineages that was demonstrated with mutant ES cells in the presence of LIF: mesendoderm and trophectoderm, not neuroectoderm.

DISCUSSION

Foxd3 Maintains ES Cell Survival and Self-renewal while Repressing Differentiation

ES cells are unique in their ability to remain self-renewing and pluripotent *in vitro*. These processes are tightly regulated by a network of transcription factors, only some of which are well understood³¹. Our results establish two intertwined roles for *Foxd3* in ES cells: this protein is required to maintain ES cell survival and self-renewal, while simultaneously repressing differentiation into either normal differentiation pathways (endoderm and mesoderm) or abnormal ones (trophectoderm) (see model in Fig. 7).

The role of *Foxd3* regulating ES cell survival is in agreement with our *in vivo* studies demonstrating similar roles for *Foxd3* in both early embryos¹¹ and neural crest progenitors¹³. *Foxd3*^{-/-} embryos exhibit increased programmed cell death at 6.5 dpc, and *in vitro* cultures of mutant blastocysts also contain more TUNEL-positive cells than wild type blastocyst cultures¹¹. Deletion of *Foxd3* in the neural crest, another multipotent cell lineage, resulted in considerable apoptosis of neural crest progenitor cells¹³. Our experiments here monitoring the expression of residual *Foxd3* in treated cells together with TUNEL revealed very few, if any, double-positive cells, strongly suggesting a cell-autonomous function of *Foxd3* in maintaining ES cell survival. Furthermore, our results show that *Foxd3* mutant ES cells maintaining Oct4 expression are protected from programmed cell death, whereas those mutant cells that have differentiated inappropriately (marked by *Foxa2*) have a much higher tendency to undergo apoptosis. These results suggest that the increased apoptosis in mutant ES cells could be a secondary consequence of aberrant differentiation. However, our results do not rule out the possibility that *Foxd3* may directly regulate genes in apoptotic pathways. Interestingly, the proliferation capacity of mutant ES cells is comparable to that of control cells even as their ability to self-renew is greatly decreased. Therefore, our results point to a specific function of *Foxd3* in maintaining ES cell survival and self-renewal, without affecting proliferation.

The early lethality of *Foxd3* mutant embryos makes it difficult to study the role of *Foxd3* in regulating differentiation¹¹. Our ES cell data presented here demonstrate up-regulation of multiple differentiation markers for several cell lineages in *Foxd3* mutant ES cells and EBs, suggesting that *Foxd3* may act as a gatekeeper in pluripotent ES cells to prevent cells from inappropriate differentiation, thereby maintaining the pool of stem cells. Similarly, in the trophoblast lineage, *Foxd3* controls differentiation of trophoblast stem cells in order to maintain a multipotent progenitor cell population¹².

Positioning *Foxd3* in the Transcription Factor Network Regulating ES Cell Self-Renewal and Pluripotency

Recent genome-wide studies suggest that Oct4, Sox2 and Nanog form a regulatory feedback circuit to maintain self-renewal and pluripotency in ES cells^{32, 33}. These are not the only crucial factors, a parallel pathway involving transcription factors *Esrrb* and *Tbx3* also represses differentiation to maintain ES cell self-renewal and pluripotency²². Expression of Oct4, Sox2 and/or Nanog in concert with Klf4, c-Myc and/or Lin-28 reprograms human or murine somatic cells from multiple sources to adopt stem cell behavior and generate iPSCs that share all the properties of ES cells⁶⁻¹⁰. In this study, we show that mRNA levels of *Pou5f1*, *Sox2*, *Nanog*, *Esrrb*, *Tbx3*, *Klf4* and *c-Myc* are maintained or slightly up-regulated in *Foxd3* mutant ES cells, suggesting that *Foxd3* acts downstream of, or in parallel with, these factors to regulate self-renewal and inhibit spontaneous differentiation of ES cells. In fact, iPSCs induced by the expression of OCT4, SOX2, NANOG and LIN28 from human somatic cells¹⁰ express FOXD3 (Dr. James Thomson, personal communication), suggesting that FOXD3 could be one of the downstream executors to induce stem cell like properties in iPSCs.

Context-Dependent Function of *Foxd3* during Development

Our results add to previous findings suggesting that *Foxd3* is a multifunctional protein playing a role in many divergent processes in the embryo and in progenitor cells. As a transcription factor, *Foxd3* has been shown to act either as a repressor or an activator depending upon the cellular context. During *Xenopus* mesoderm induction, *FoxD3* functions as a repressor to induce mesoderm and elegant structure-function analyses identified a strong transcriptional repression domain in the C terminus of *Xenopus* *FoxD3*^{34, 35}. Within this domain is a Groucho corepressor interaction motif conserved in *Xenopus*, zebrafish, chick and mammals. In *Xenopus*, *Foxd3* binds DNA and recruits Grg4 (Groucho-related gene 4) to repress

transcription³⁵, likely by the recruitment of histone deacetylases that alter chromatin conformation and result in decreased transcription. It is unclear if mammalian Foxd3 functions in the same manner as *Xenopus* FoxD3; mammalian Foxd3 has several divergent sequence domains from either *Xenopus* or zebrafish FoxD3 which might suggest that these orthologs function by different mechanisms. Expression of the murine homologs of *Grg1-4*, called *Tle1-4*, have been implicated in a variety of embryonic functions including segmentation and neurogenesis (reviewed in Buscarlet and Stifani, 2007 and Gasperowicz and Otto, 2005)^{36, 37}, but thus far a role for these proteins in ES cell pluripotency has not been examined. Embryonic expression of the murine Groucho homologs is widespread making it likely that other co-factors might also be relevant to the regulation of Foxd3 protein function in a context specific manner. Polycomb group (PcG) transcriptional repressors repress many developmental regulatory genes in murine ES cells³⁸. Because neither Groucho nor PcG proteins bind DNA directly, it is likely that they are recruited to target genes by sequence-specific DNA-binding transcription factors such as Foxd3.

In many circumstances Foxd3 acts as a repressor, but in other situations, Foxd3 has transcriptional activator activity. In the human embryonic kidney 293 cell line, ectopically expressed Foxd3 activates expression of the endoderm genes *Foxa1* and *Foxa2*³⁹. Other work demonstrated that Foxd3 activates expression of a reporter driven by either the *Nanog* or *Pou5f1* promoter in ES cells, and ES cells transfected with siRNA for *Foxd3* have reduced expression of *Nanog* and *Pou5f1*⁴⁰. These results support an activator role for Foxd3 maintaining *Pou5f1* and *Nanog* expression, however, co-transfection of Foxd3 and a reporter preceded by multimers of a 30bp Foxd3 binding site from the *Nanog* promoter, *Foxd3* overexpression causes decreased expression of this reporter in a dose-dependent manner, suggesting that Foxd3 can act as a repressor in ES cells⁴⁰. ES cells transfected with Foxd3 siRNA show reduced expression of *Pou5f1* and *Nanog*, but the efficiency of the siRNA is unknown. Both Oct4 and Sox2 levels must be maintained in a very narrow window to maintain pluripotency of ES cells, and Oct4 and *Nanog* have a complex co-regulation with biphasic regulation of *Nanog* by Oct4, such that a steady-state concentration of Oct4 maintains *Nanog* expression, while an elevated level of Oct4 represses *Nanog* mRNA levels⁴⁰. Therefore, it is clear that the cross-regulation of these self-renewal factors is complex, and individual transcription factors may function as repressors or activators in the same cell type.

These proposed divergent mechanisms of action for Foxd3 as either a repressor or an activator could indicate a role for Foxd3 as a convergent point for multiple pathways. Other Fox proteins have been proposed to function as so-called “pioneer factors” to modify chromatin; FoxA1 binds the *Albumin1* enhancer prior to *Alb1* transcription and this binding of FoxA1 appears to open the chromatin and allow access to other transcription factors^{41, 42}. Similar function has been ascribed to Foxe1 binding to the thyroperoxidase promoter and thereby opening chromatin⁴³. It is possible that Foxd3 is acting at promoters of differentiation specific genes to allow access to other transcription factors that modulate transcription. In fact, this has been suggested by chromatin immunoprecipitation assays demonstrating that Foxd3 binds the inactive *Alb1* promoter in ES cells and may modulate methylation of a CpG to control chromatin conformation⁴⁴. This is supported by our data in Foxd3 mutant ES cells that along with other markers of endoderm development, *Alb1* transcription is precociously increased in EBs and raises the possibility that Foxd3 may be acting at a key step in regulating multiple early lineage loci. Future work will be focused on identifying the molecular partners and targets of Foxd3 that function to carry out these essential roles in both ES cells and other multipotent precursors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Tamoxifen treatment induces efficient deletion of *Foxd3*

(A): Diagram of the *Foxd3* wild type, conditional ("floxed") and recombined alleles. Arrows indicate PCR primers that flank the 5' LoxP site. (B): Genomic DNA PCR of wild type ES cells, *Foxd3^{fl/fl}; Cre-ER* and *Foxd3^{fl/fl}* ES cells cultured in the presence or absence of 2 μ M TM for 3 days using flox and Cre primers. HPRT serves as an internal control. (C): *Foxd3*, *Cre* and *Hprt* RT-PCR of cDNA from multiple lines of ES cells treated with or without 2 μ M TM for 3 days. (D): qRT-PCR showing significant reduction of *Foxd3* mRNA in *Foxd3^{fl/fl}; Cre-ER* ES cells treated with 2 μ M TM. Levels of *Foxd3* transcript are compared by Student's t-test (***: $p < 0.001$). (E): Immunocytochemistry of Foxd3 (red) and DAPI (blue) showing absence

of nuclear Foxd3 in most TM-treated *Foxd3^{fl/fl}*; *Cre-ER* ES cells with rare retention of Foxd3 in the nuclei of some cells (arrowheads). Scale bar: 50µm.

Figure 2. Defective self-renewal of *Foxd3* mutant ES cells

(**A**): Cell number analysis of *Foxd3^{fl/fl}; Cre-ER* cell lines and *Foxd3^{fl/fl}* control cell lines treated with 0, 2 or 4μM TM. Surviving cells were counted with a hemocytometer after 2, 3 and 4 days of TM treatment. (**B**): Alkaline phosphatase staining and Foxd3 immunolocalization in colonies generated from the self-renewal analysis of *Foxd3^{fl/fl}; Cre-ER* cell lines. Scale bar: 50μm. (**C**): Colony formation efficiency as shown by the number of AP-positive colonies normalized against total number of cells plated. (**D**): Number of cells per colony in control and mutant self-renewal cultures.

Data shown are Mean±SEM (***) $p < 0.001$.

Figure 3. Foxd3 is required for ES cell survival

(A): TUNEL labeling (red) and Foxd3 staining (green) of *Foxd3^{fl/fl}; Cre-ER* and *Foxd3^{fl/fl}* cell lines with or without 2 μ M TM treatment revealing increased apoptosis of Foxd3-deficient ES cells (arrows). (B): Quantification of TUNEL-positive cells normalized against total number of DAPI-stained cells in *Foxd3^{fl/fl}; Cre-ER* cultures after 2 or 3 days of TM treatment. (C): BrdU incorporation as a measure of cells in S phase (red) and Foxd3 (green) staining. Many *Foxd3*-deficient ES cells are BrdU-positive (arrows). (D): Quantification of percentage of BrdU-positive cells in *Foxd3^{fl/fl}; Cre-ER* cell cultures. (E): Phospho-histone H3 (red) staining for cells in G2-M phase. (F): Quantification of percentage of pH3-positive cells in *Foxd3^{fl/fl}; Cre-ER* cell cultures. ES cells in (A), (C) and (E) were counter-stained with DAPI (blue).

Arrowheads in (A) and (C) point to a few Foxd3-positive cells in mutant cultures. Scale bar: 50 μ m. Data in (B), (D) and (F) were obtained from three independent experiments with more than 7000 DAPI-positive cells counted for each treatment, and are shown as Mean $\pm$ SEM (***) p<0.001).

Figure 4. *Foxd3* is not required for the expression of stem cell markers

(A): Immunocytochemistry shows nuclear staining of Oct4, Sox2 and Nanog (red) in *Foxd3^{fl/fl}; Cre-ER* ES cells with or without 2μM TM treatment for 3 days. ES cells were counter-stained with DAPI (blue). Oct4 (red) and Foxd3 (green) double staining (leftmost panel) reveals that most *Foxd3*-deficient ES cells are Oct4-positive in mutant cultures and very little Foxd3 protein is observed in treated cells. Scale bar: 50μm. (B): qRT-PCR analysis of stem cell marker gene expression in 2μM TM (red line) or no TM (blue line) treated cells for 1-3 days. mRNA levels of each gene are compared between control and TM-treated cells by Student's t-test (***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$).

Figure 5. Foxd3 represses ES cell differentiation towards multiple lineages

(A): qRT-PCR analysis of transcript levels of primitive endoderm markers *Foxa2*, *AFP* and *Sox17* in ES cells cultured with or without TM for 3 days. Bars represent transcript levels for each gene in 2μM TM treated cells, and horizontal lines indicate the mRNA levels in untreated ES cells arbitrarily designated as 1. (B): qRT-PCR analysis of mRNA levels of trophoctoderm markers *Cdx2*, *Fgfr2* and *Csh1/PLI*. (C): qRT-PCR analysis of mRNA levels of epiblast marker *Fgf5*, mesendoderm markers *T* and *Gsc*. (D): qRT-PCR analysis of neurectoderm markers *Sox1*, *Nestin*, and *Pax6*. (E): Immunocytochemistry of Foxa2 (red) and either Sox2 or Oct4 (green) in *Foxd3^{fl/fl}; Cre-ER* ES cells treated with 2μM TM for 3 days. Arrowheads: Sox2/Foxa2 double-positive cells. Scale bar: 50μm. (F): Percent of Oct4- or Foxa2-positive cells in control and 2μM TM-treated cultures. Data were collected from three independent experiments, and > 2000 cells were counted for each treatment group. (G): TUNEL labeling (green) and immunocytochemistry for either Oct4 or Foxa2 (red) in *Foxd3^{fl/fl}; Cre-ER* ES cells treated with 2μM TM for 3 days. Arrowheads: TUNEL/Foxa2 double-positive cells that are also DAPI-positive. Arrows: TUNEL/Foxa2 double-positive cells that are DAPI-negative indicating they have lost their nuclear integrity. Scale bar: 25 μm. (H): Percent of TUNEL-positive cells in Oct4-positive or Foxa2-positive populations in control and mutant cultures. More than 2000 cells were counted for each treatment group. Data shown are Mean±SEM (***) $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

Figure 6. Increased differentiation of *Foxd3* mutant EBs

(A): qRT-PCR analysis of endoderm marker *Foxa2* mRNA levels in D2-D8 EBs cultured in the presence (red) or absence (blue) of 2μM TM. (B): Sections of untreated and TM-treated D4 and D8 EBs that have undergone whole mount immunocytochemistry for Foxa2 (red) and counter-staining with DAPI (blue). Scale bar: 100μm. (C): Percent of Foxa2-positive cells in D4 and D8 EBs. (D-I): qRT-PCR analysis of transcript levels of endoderm markers *Sox17*, *AFP* and *Alb1*, trophoblast marker *Cdx2*, mesoderm marker *Gsc* and neuroectoderm marker *Sox1* in D2-D8 EBs. Graphs in (A), (C)-(I) were plotted in logarithmic scale. mRNA levels of each gene are compared between control and TM-treated cells at each stage by Student's t-test. Data shown are Mean±SEM (***) $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

Figure 7. Model of Foxd3 functions in murine ES cells

Data presented here suggest a role for Foxd3 in maintenance of ES cell self-renewal and survival, and inhibition of differentiation into multiple lineages.