Requirement for *Foxd3* in maintaining pluripotent cells of the early mouse embryo

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Critical to our understanding of the developmental potential of stem cells and subsequent control of their differentiation in vitro and in vivo is a thorough understanding of the genes that control stem cell fate. Here, we report that *Foxd3*, a member of the forkhead family of transcriptional regulators, is required for maintenance of embryonic cells of the early mouse embryo. *Foxd3*–/– embryos die after implantation at approximately 6.5 days postcoitum with a loss of epiblast cells, expansion of proximal extraembryonic tissues, and a distal, mislocalized anterior organizing center. Moreover, it has not been possible to establish *Foxd3*–/– ES cell lines or to generate *Foxd3*–/– teratocarcinomas. Chimera analysis reveals that *Foxd3* function is required in the epiblast and that *Foxd3*–/– embryos can be rescued by a small number of wild-type cells. *Foxd3*–/– mutant blastocysts appear morphologically normal and express *Oct4*, *Sox2*, and *Fgf4*, but when placed in vitro the inner cell mass initially proliferates and then fails to expand even when Fgf4 is added. These results establish *Foxd3* as a factor required for the maintenance of progenitor cells in the mammalian embryo.

[*Keywords*: mouse embryogenesis; stem cells; *Foxd3*; winged helix gene]

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Multipotent progenitor cells exist transiently in the mammalian embryo in several tissues including the preimplantation stage blastocyst, the gastrulating epiblast, and the neural crest. Each of these cell types can be cultured in vitro to generate multipotent stem cell lines, and when grafted ectopically, blastocysts and epiblast tissue will produce teratocarcinomas containing multipotent stem cells. Understanding the common molecular regulatory mechanisms of different stem cell types is critical to an understanding of how multipotency is maintained in vivo and how differentiation is controlled.

Mammalian embryonic development initiates with a series of cleavage divisions, and at 2.5 days postcoitum (dpc) the free-floating mouse embryo undergoes compaction: cell boundaries become tightly apposed to one another and cells are no longer equivalent. Inner cells contribute to the inner cell mass (ICM) and embryo proper, whereas outer cells contribute to the trophectoderm, a tissue essential for implantation. After implantation, the ICM proliferates and extends distally to form the cup-like egg cylinder (at ~5.5 dpc) consisting of an inner layer of epiblast cells and an outer layer of extraembryonic visceral endoderm (for review, see Hogan et al. 1994).

Few genes have been identified that are required for the maintenance of the epiblast cell population and the establishment of ICM-derived embryonic stem (ES) cells in vitro. *Oct4*–/– embryos, mutant for this POU family transcription factor, die around 5.0 dpc, shortly after implantation but before the egg cylinder is formed. *Oct4*–/– ES cells cannot be established, as the ICM cells fail to survive (Nichols et al. 1998). Survival of multipotent cells of the ICM is highly sensitive to Oct4 expression levels; low Oct4 levels result in the differentiation of ES cells into trophoblast, and high levels result in differentiation into primitive endoderm and mesoderm (Niwa et al. 1998). Fibroblast growth factor 4 (Fgf4) is also required for formation of the egg cylinder; *Fgf4*–/– embryos fail to develop after implantation, and cells of the ICM do not proliferate in vitro (Feldman et al. 1995). *Fgf4* transcription is directly regulated by Oct4 and Sox2 binding to an *Fgf4* enhancer element (Yuan et al. 1995). Establishment of *Fgf4*–/– ES cells is possible with addition of recombinant Fgf4 to the culture medium (Wilder et al. 1997). The complexities of maintaining pluripotent cells in the embryo are likely to involve additional key regulatory factors.

Foxd3 (previously published as *Hfh2*) was isolated in a

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low-stringency screen for forkhead family genes (Labosky and Kaestner 1998). Originally termed "*Genesis,*" *Foxd3* was reported to be expressed only in mouse ES cells and their malignant equivalents (Sutton et al. 1996). In addition, *Foxd3* expression is one of several diagnostic markers characteristic of human ES cell lines (M.F. Pera, pers. comm.). However, *Foxd3* expression is not as restricted as first suggested and is also detected during early embryogenesis in the epiblast and later in neural crest cells (Labosky and Kaestner 1998; Hromas et al. 1999; Dottori et al. 2001). *Foxd3* has been implicated in the control of differentiation in multiple systems: Overexpression of *Foxd3* in a myeloid cell line prevents appropriate maturation of these cells into granulocytes (Xu et al. 1998), ectopic expression of *Foxd3* in the chick neural tube changes the fate of those cells into neural crest and can interfere with the subsequent differentiation of crest derivatives (Dottori et al. 2001; Kos et al. 2001), and *Foxd3* mRNA can induce the formation of mesoderm in *Xenopus* (D.S. Kessler, unpubl.). We demonstrate here that *Foxd3* is required for the maintenance of pluripotent cells in the preimplantation and peri-implantation stages of mouse embryogenesis.

Results

Expression of Foxd3 *in early mouse embryos*

Foxd3 was previously shown to be expressed in premigratory neural crest cells, and expression is downregulated in all differentiated derivatives with the exception of Schwann cells (Labosky and Kaestner 1998; Dottori et al. 2001), a cell type that can give rise to multipotent stem cells in vitro (Stemple and Anderson 1992). To assess *Foxd3* expression at earlier stages of development, we used RT-PCR to analyze expression in unfertilized oocytes through early implantation-stage embryos. *Foxd3* is not expressed in the unfertilized oocyte or fertilized one-cell embryos, but transcripts are detected in blastocyst-stage embryos, after the time when zygotic transcription is initiated in the mouse embryo (Fig. 1a). Whole mount and section in situ hybridization detected expression of *Foxd3* throughout the epiblast of the 6.5-dpc embryo with faint expression in the extraembryonic region (Fig. 1b, data not shown). The extraembryonic expression was confirmed by RT-PCR analysis of dissected extraembryonic and embryonic portions of embryos (Fig. 1a).

Targeted deletion of the Foxd3 *locus*

To determine the role of *Foxd3* during embryogenesis, we replaced the entire coding region with a *neo* resistance cassette and a *Gfp* gene, generating a null mutation (Fig. 1c–e). No *Foxd3* mRNA was detected in *Foxd3*–/– embryos, confirming that this is a null mutation (Fig. 1a). Southern analysis with 5′ (data not shown) and 3′ (Fig. 1d) flanking probes confirmed the structure of the targeted allele as shown. The *neo* cassette was flanked by *loxP* sites for subsequent CRE deletion. We generated three alleles of *Foxd3*: the first with an insertion of a *Gfp* gene with an internal ribosomal entry site (*Ires-Gfp*), the second with a *Gfp* gene insertion, and the third with the *Ires-Gfp* with the *neo* cassette removed. All three alleles of *Foxd3* ($Foxd3^{Ires\text{-}Gfp}$, $Foxd3^{Gfp}$, and $Foxd3^{Ires\text{-}Gfp\Delta neo}$) gave the same phenotype, and most of the studies reported here were carried out with the $Foxd3^{Ires\text{-}Gfp}$ allele, hereafter called *Foxd3*–.

Embryonic lethality of Foxd3–/– *embryos*

Foxd3+/– mice derived from targeted ES cell lines were normal and healthy, but no *Foxd3*–/– mice were born from *Foxd3*+/– intercrosses with over 300 mice weaned and analyzed, indicating that *Foxd3*–/– embryos did not survive to birth. Timed matings were carried out and no *Foxd3*–/– embryos were detected at 13.5 dpc; abnormal embryos consisting primarily of an empty yolk sac were present at 9.5 dpc, and PCR genotyping revealed these to be *Foxd3*–/– (data not shown). Morphological and histological analysis at 5.5 dpc revealed no overtly abnormal embryos in multiple litters. Further analysis revealed that abnormalities in *Foxd3*–/– embryos were apparent as early as 6.5 dpc (Figs. 2, 3, see below). At this time, *Foxd3*–/– embryos do not have a smooth rounded distal end characteristic of wild-type embryos. Instead, cells resembling extraembryonic endoderm extend in multiple finger-like protrusions at the distal tip (Fig. 2, cf. a,i and b,j). Normally, the proximal extraembryonic endoderm is columnar with large vacuoles, whereas distal extraembryonic endoderm is squamous in character (Fig. 2c,e). In *Foxd3*–/– embryos, this distinction is not seen; columnar and vacuolated extraembryonic endoderm extends nearly to the distal tip of the mutant embryo (yellow arrows in Fig. 2d,f). Significantly, the epiblast of *Foxd3*–/– embryos is reduced in size, and histological analysis revealed the absence of a primitive streak. These observations suggest that either the epiblast is converted to extraembryonic tissue or that upon failure of the epiblast the extraembryonic tissue expands distally.

To determine whether loss of epiblast was a result of decreased cell proliferation, we used histone H3 phosphorylation to assess cells in M phase. A marked decrease in M-phase cells was observed in the inner and distal cells of mutant embryos (Fig. 2g,h). To quantify these results, we counted positive cells located in the internal portion of 6.5-dpc embryos but excluded from the external layer of extraembryonic endoderm. The number of phospho-H3-positive cells in normal 6.5-dpc embryos averaged 6.53 (n = 17 embryos) versus 2.64 (n = 22 embryos) in abnormal (presumably *Foxd3*–/–) embryos. A two-tailed T-test demonstrated that this difference is statistically significant with $P < 0.05$. There was a similar decrease in the number of S-phase cells as shown by BrdU incorporation (data not shown). TUNEL labeling showed a slight increase in cell death in the 6.5-dpc distal embryo (Fig. 2i,j), but by 7.5 dpc (head fold stage), the entire distal region of the mutant embryo was

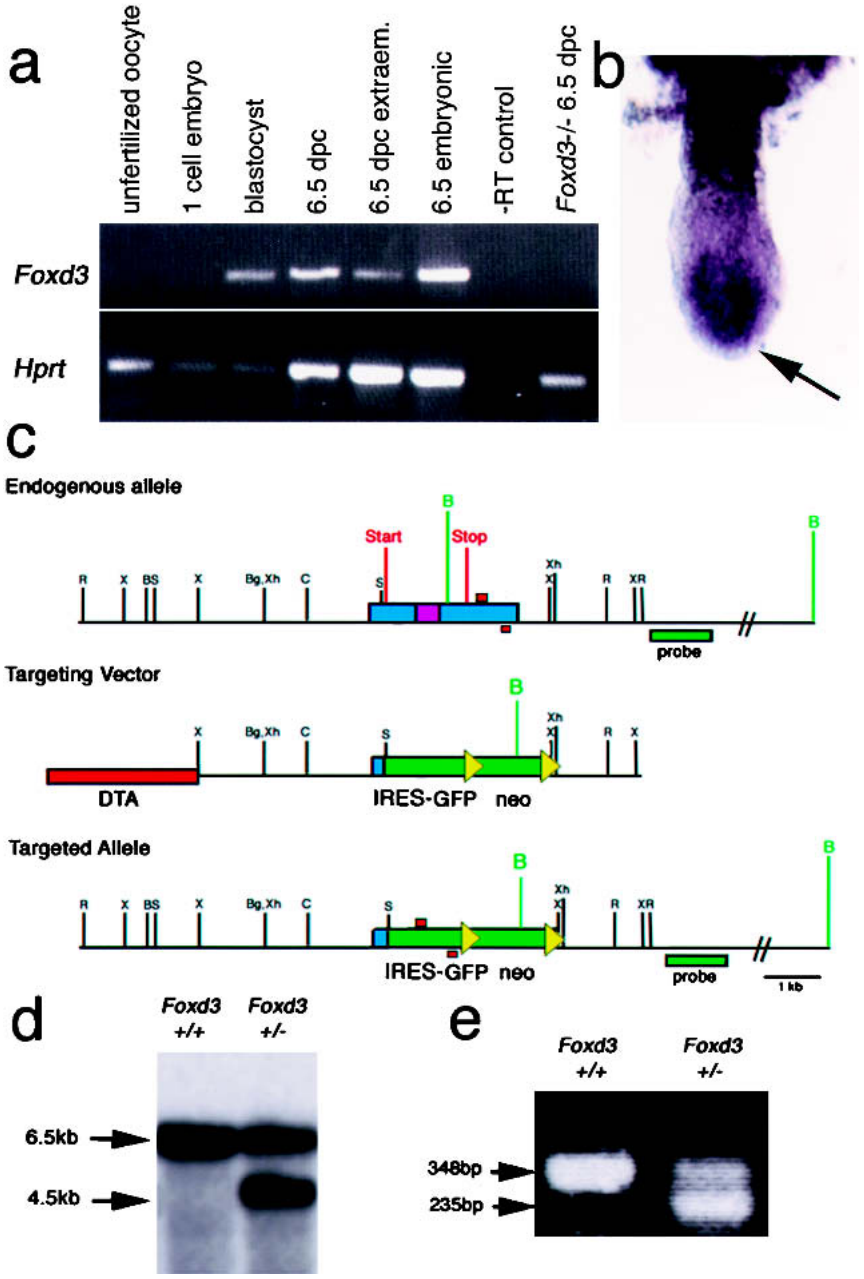

Figure 1. Embryonic expression of *Foxd3* and generation of *Foxd3* mutant allele. (*a*) RT-PCR analysis of staged mouse embryos shows that *Foxd3* is not expressed in unfertilized oocytes or one-cell embryos but can be detected in the blastocyst at 3.5 dpc. *Foxd3* mRNA is also detected at 6.5 dpc in both the extraembryonic and the embryonic portions of the embryo. Expression is undetectable in a 6.5-dpc *Foxd3*–/– embryo. Negative controls with no reverse transcriptase added show no amplification of the *Foxd3* message. RT-PCR for the *hypoxanthine phosphoribosyl transferase* gene (*Hprt*) was used to monitor the integrity of the cDNA produced by reverse transcription. (*b*) Whole mount in situ hybridization of a 6.5-dpc embryo shows that *Foxd3* transcripts are present in the layer of epiblast cells, but not in the outer layer of endoderm (arrow). (*c*) The *Foxd3* null mutation was generated using a targeting vector shown here. PCR primers for genotyping are shown as red boxes, and the external 3′ probe used to screen ES cells is shown in green. The *loxP* sites flanking the *neo* cassette are yellow triangles. (*d*) Southern analysis of ES cells was performed by digesting genomic DNAs with *BamH*1 as diagrammed in *c*. (*e*) PCR was used to genotype *Foxd3* mice and embryos.

severely truncated and thus cell death measurements were not informative (Fig. 2k,l). Together, these data indicate that cell proliferation is reduced and cell death is increased in the distal embryonic portion of 6.5-dpc *Foxd3*–/– embryos compared to the wild type.

Molecular analysis of Foxd3–/– *embryos*

To define the molecular basis of the developmental defects, we analyzed lineage-specific gene expression (Fig. 3). In all of the panels of Figure 3, wild-type expression of the marker analyzed is shown on the left with *Foxd3*–/– embryos to the right. Because ectopic expression of *Foxd3* in *Xenopus* embryos (D. Kessler, unpubl.) can cause the formation of mesoderm and our histological analyses of *Foxd3*–/– embryos indicated a disruption in the primitive streak, we first wished to determine whether any mesoderm was present in *Foxd3*–/– embryos. Expression of the *T* gene (*Brachyury*), normally expressed in mesodermal cells (Herrmann 1991), was not detected in early or late 6.5-dpc mutant embryos (Fig. 3a), suggesting that mesodermal cells are not being generated and that gastrulation is not occurring. The absence of expression of other embryonic and extraembryonic mesoderm markers, including *Wnt3*, *Mm1*, *Eomesodermin*, *Fgf8*, *Bmp4*, and *Mesp1* (Fig. 3b–g; Crossley and Martin 1995; Saga et al. 1996; Lawson et al. 1999; Liu et al. 1999; Pearce and Evans 1999) supports this conclusion.

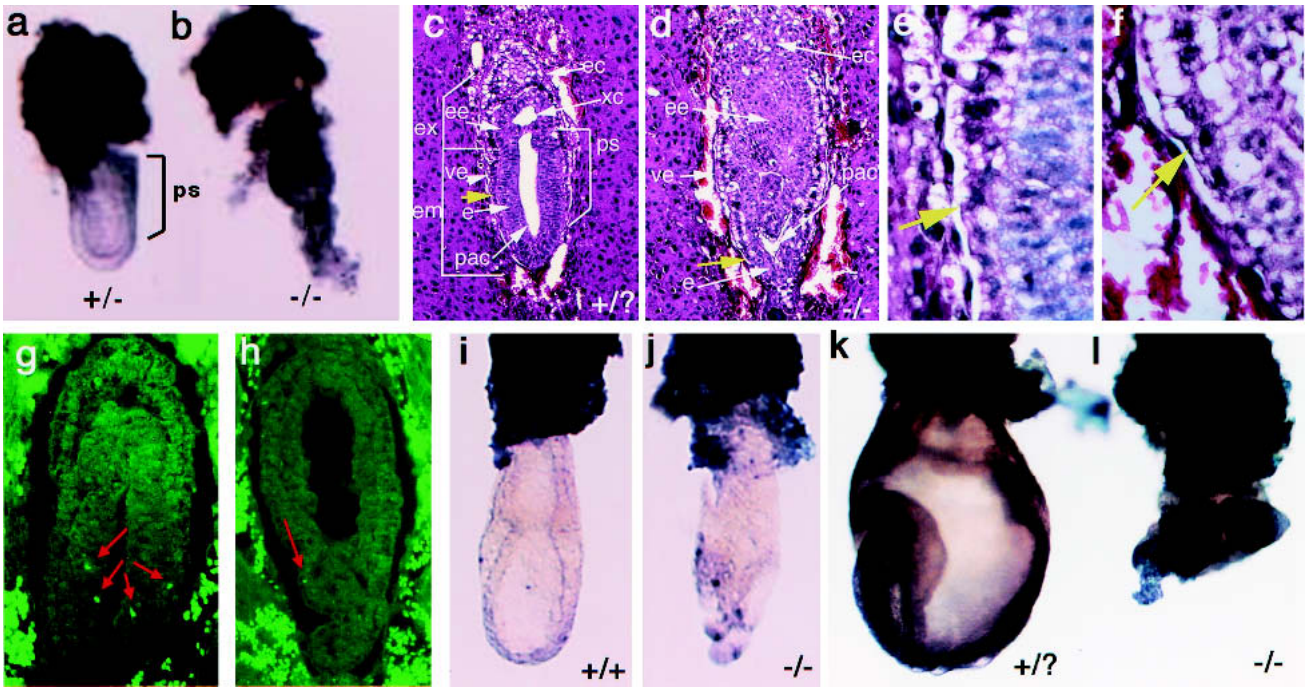

Figure 2. Gastrulation of *Foxd3*–/– embryos is disrupted. (*a*,*b*) Morphology of embryos dissected at 6.5 dpc shows that *Foxd3*–/– embryos have an abnormal distal epiblast region and no evidence of a primitive streak. (*c*) A sagittal section of a normal 6.5-dpc embryo from a *Foxd3*+/– intercross. Note the clear distinction between the extraembryonic (ex) and embryonic (em) regions. The proamnionic cavity (pac) extends the length of the embryonic region, while the exocoelomic cavity (xc) is restricted to the extraembryonic region. (*d*) A sagittal section of an abnormal 6.5-dpc embryo from the same cross. Remnants of a proamniotic cavity are visible, and visceral endoderm (ve), extraembryonic ectoderm (ee), and embryonic ectoderm (e) can be identified. (*e*,*f*) The normal and abnormal visceral endoderm in *c* and *d* are shown at higher magnification. The region of the section is indicated in *c* and *d* with yellow arrows. (*g*,*h*) Phosphohistone H3 is detected at 6.5 dpc, and although several epiblast cells of the normal embryo are strongly stained, no internal distal cells of a *Foxd3*–/– embryo react with the antibody for phosphohistone H3. Positive cells are indicated with red arrows. (*i*,*j*) TUNEL staining to detect cells undergoing apoptosis shows no appreciable cell death at 6.5 dpc in a normal embryo, with some cell death in a *Foxd3*–/– embryo. By 7.5 dpc (*k*,*l*), there is very little embryonic tissue remaining in the *Foxd3*–/– embryo. e, embryonic ectoderm; ec, ectoplacental cone; ee, extraembryonic ectoderm; em, embryonic region; ex, extraembryonic region; pac, proamnionic cavity; ps, primitive streak; ve, visceral endoderm; xc, exocoelomic cavity.

Pou5f1, encoding the transcription factor Oct4, is expressed throughout the epiblast in wild-type embryos and is essential for the survival of epiblast cells (Rosner et al. 1990; Nichols et al. 1998). Despite the absence of morphological differences at 5.5 dpc, *Pou5f1* expression was not detected in 22% of embryos dissected from *Foxd3*+/– intercrosses (Fig. 3h; total number of embryos = 27). At 6.5 dpc, most *Foxd3*–/– embryos showed no expression of *Pou5f1*, although weak expression in cells scattered throughout the embryo was sometimes observed (Fig. 3h, slight staining in far right embryo). *Otx2* is normally expressed throughout the epiblast and gradually becomes restricted to the anterior half of the embryo (Simeone et al. 1993). In *Foxd3*–/– 6.5-dpc embryos, there was no *Otx2* expression (Fig. 3i), but in embryos a few hours older (~6.75 dpc) a few *Otx2*-expressing cells were present at the distal tip (Fig. 3i, far right embryo). To examine *Nodal* expression in mutant embryos, we generated *Foxd3*–/–; *Nodal-lacZ*+/– embryos. Using the *Nodal-lacZ* allele, β-galactosidase activity is normally detected throughout the epiblast (Varlet et al. 1997; Brennan et al. 2001), but no β-galactosidase activity was detected in most *Foxd3*–/– embryos, with an occasional embryo (2 of 15) showing β-galactosidase activity at the distal tip (arrow in Fig. 3j). No epiblast expression of the Nodal cofactor *Cripto* was detected (Fig. 3k; Ding et al. 1998).

In contrast to the loss of epiblast and mesodermal gene expression, distal expansion of the expression of extraembryonic endodermal markers *Pem* and *Amn* (Lin et al. 1994; Kalantry et al. 2001) was observed at 6.5 dpc (Fig. 3l,m), as was expression of the extraembryonic ectodermal markers *Bmp8b*, *Fgfr2*, and *Err*β (Fig. 3n–p). Formation of the anterior visceral endoderm (AVE), a signaling center required to pattern the anterior-posterior axis of the embryo, was also examined. Expression of the AVE markers *Lim1* and *Hex* (Barnes et al. 1994; Thomas et al. 1998) was restricted to the distal tip of *Foxd3*–/– embryos (Fig. 3q,r). This result suggests that the occasional distal expression of *Otx2* and *Nodal* at the tip of *Foxd3*–/– embryos (Fig. 3i,j) is most likely due to the ectopic position of the AVE. These observations demonstrate that in *Foxd3*–/– embryos, the AVE is specified but fails to move anteriorly in the absence of the epiblast and

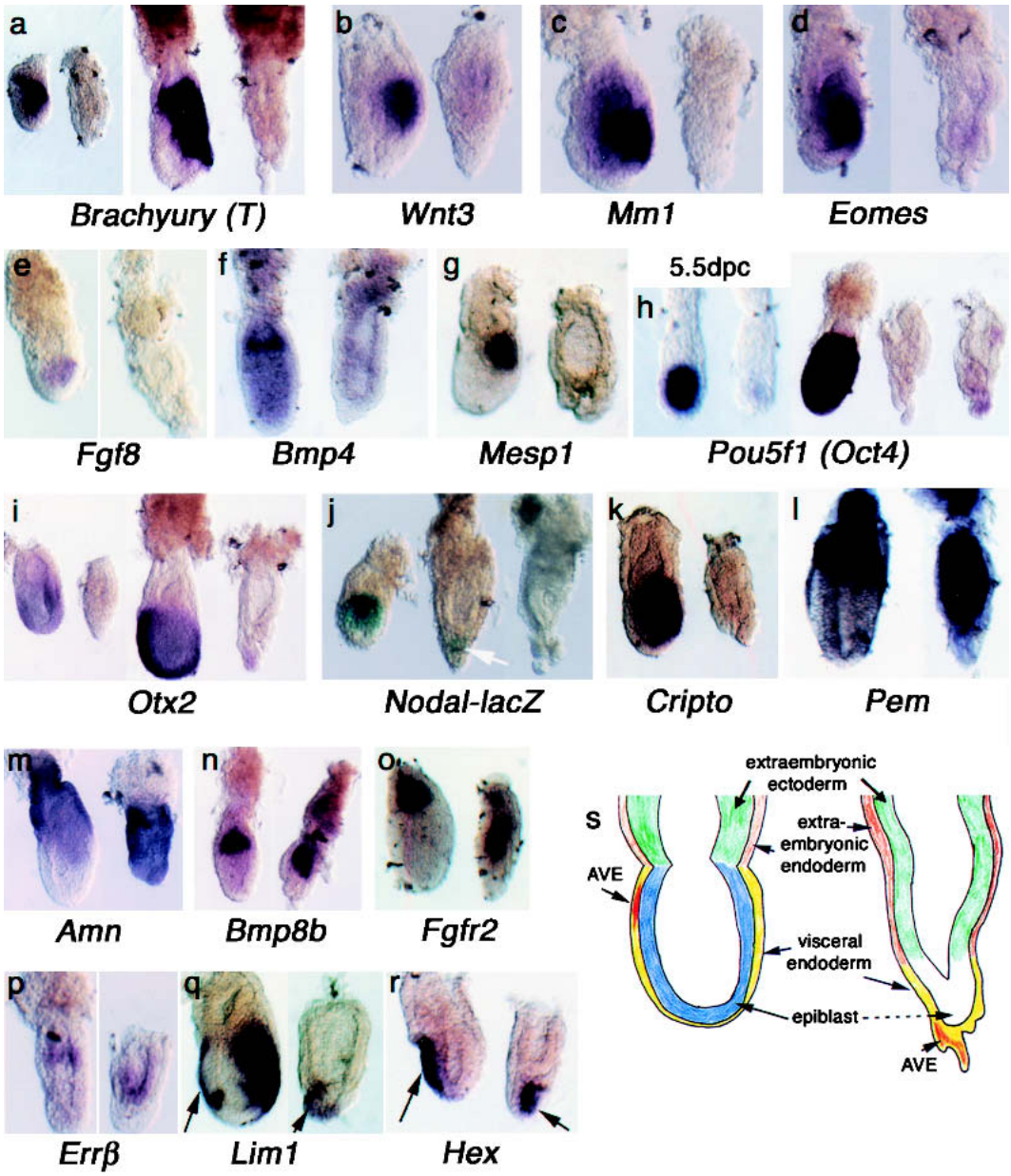

Figure 3. Molecular marker analysis of *Foxd3* mutant embryos. Whole mount in situ analysis of normal littermates is shown on the left side of each set of embryos. All embryos are 6.5 dpc except where noted. Anterior (when discernible) is to the *left*. (*a*) *Brachyury* (or *T*) is expressed in the proximal epiblast of early 6.5-dpc embryos and in all cells of the primitive streak at later stages. *T* expression is never detected in *Foxd3*–/– embryos. (*b*) *Wnt3*, also normally expressed in the proximal epiblast and later in the primitive streak, is not expressed in mutants. (*c–e*) *Mm1*, *Eomes*, and *Fgf8*, markers of mesodermal cells of the primitive streak, are not expressed in *Foxd3*–/– embryos. (*f,g*) Extraembryonic mesodermal markers *Bmp4* and *Mesp1* are not detected in mutant embryos. (*h*) *Pou5f1/Oct4* expression is detected throughout the epiblast in a normal embryo at 5.5 dpc, but in *Foxd3*–/– embryos, expression is missing at 5.5 dpc. At 6.5 dpc, *Pou5f1/Oct4* is expressed robustly in normal embryos (*middle* embryo) but is either missing or only weakly expressed in scattered cells (the two embryos on the *right*). (*i*) *Otx2* expression is normally detected throughout the epiblast of the early streak-stage embryo, but is missing in mutant littermates (first pair of embryos). *Otx2* expression in an early headfold-stage embryo (~7 dpc) is concentrated in the anterior half of the embryo (third embryo). A small amount of *Otx2* expression is detected in the distal tip of the mutant littermate (*far right* embryo). (*j*) *Nodal-lacZ* expression is detected in the proximal epiblast of the early streak-stage embryo (*left* embryo). However, *Nodal-lacZ* expression was not detected in most *Foxd3*–/– embryos (*far right* embryo). An exception is shown here with a low level of β-galactosidase activity restricted to the distal tip (arrow, *middle* embryo). (*k*) The Nodal cofactor *Cripto* is not expressed in mutant embryos. (*l,m*) Extraembryonic visceral endoderm, as indicated by *Pem* and *Amn*, is distally expanded in the mutants. (*n–p*) Extraembryonic ectoderm is also distally expanded as shown by expression of *Bmp8b*, *Fgfr2*, and *Err*β. (*q,r*) The AVE signaling center, indicated by the arrows, is located distally in *Foxd3*–/– embryos as shown by the expression of *Lim1* and *Hex*. The posterior *Lim1* expression in *q* corresponds to the mesodermal expression domain of this gene. (*s*) Schematic illustrating the morphological changes in *Foxd3*–/– embryos.

primitive streak. As summarized in Figure 3s, the data show that a loss of *Foxd3* function results in a dramatic reduction in cells of the epiblast and their derivatives, a distal expansion of proximal extraembryonic tissues, and a mislocalization of the AVE. This suggests that *Foxd3* plays a critical role in maintaining the number of epiblast cells and therefore in establishing the proximal-distal patterning in the early embryo.

Foxd3 *is not required for maintenance of* Oct4 *or* Fgf4 *expression in blastocysts*

The POU homeobox gene *Pou5f1*, encoding Oct4 protein, is required for the identity of pluripotent progenitor cells in the ICM of the blastocyst. Embryos lacking Oct4 die around 5.5 dpc, prior to the formation of the egg cylinder (Nichols et al. 1998). *Oct4* mRNA is not expressed in *Foxd3*–/– embryos as early as 5.5–6.5 dpc (Fig. 3a), but because Oct4 function is required before this time, we examined *Foxd3*–/– embryos for expression of this key regulatory gene prior to implantation. The secreted protein Fgf4 is also required early in the peri-implantation mouse embryo, and Oct4 and Sox2 proteins have been shown to regulate *Fgf4* expression directly (Yuan et al. 1995). We therefore also examined *Sox2* and *Fgf4* mRNAs in *Foxd3*–/– blastocysts.

Blastocysts were harvested from *Foxd3*+/– intercrosses and individually processed for RT-PCR. Expression of the *hypoxanthine phosphoribosyl transferase* (*Hprt*) gene was used as a positive control, and only *Hprt*-positive samples were used for subsequent analyses. Expression of *Foxd3* in blastocysts was used to genotype the samples, and *Foxd3*-negative/*Hprt*-positive samples were scored as *Foxd3*–/– blastocysts. *Oct4*, *Sox2*, and *Fgf4* mRNAs were detected in all blastocysts, regardless of *Foxd3* expression (Fig. 4a). *Oct4* mRNA was localized to the ICM in all blastocysts from intercrosses (Fig. 4b; n = 18). In addition, immunofluorescent localization of Oct4 in blastocysts harvested from intercrosses showed that 100% of the 23 blastocysts had robust nuclear staining of Oct4 protein (Fig. 4c). Therefore, expression of several genes known to be required for early ICM proliferation and expansion are initiated and maintained in the absence of *Foxd3*.

Foxd3 *function is required in the epiblast*

To determine whether *Foxd3* function is required in the epiblast or in extraembryonic trophoblast and endodermal tissues, we took advantage of the developmental restriction of ES cells. ES cells injected into a recipient blastocyst contribute almost exclusively to epiblast derivatives and not extraembryonic tissues (Beddington and Robertson 1989). *Rosa26.1* ES cells were injected into blastocysts from *Foxd3*+/– intercrosses, and all chimeras were dissected between 8.5 and 9.5 dpc; none were allowed to progress further in development. Because ES cells do not contribute to the extraembryonic endoderm, we used this tissue to genotype recipient blastocysts retrospectively. We generated 140 embryos in these experiments, and retrospective genotyping indicated that of the recipient blastocysts, 43 were +/+, 81 were +/–, and 10 were –/– (six embryos could not be genotyped). Two examples of chimeras obtained from *Foxd3*–/– blastocysts are shown in Figure 5a–d. Of the 10 embryos obtained from *Foxd3*–/– blastocysts, eight were morphologically normal and two recapitulated the *Foxd3*–/– phenotype. Staining for β-galactosidase activity demonstrated that most rescued chimeras (5 of 8) contained a large portion of wild-type cells such as the chimera shown in Figure 5b. However, even a low (~25%) contribution of wild-type ES cells (embryo shown in Fig. 5a) completely rescued the *Foxd3*–/– defects. The remaining two embryos derived from *Foxd3*–/– blastocysts were morphologically identical to *Foxd3*–/– embryos and had no contribution of wild-type ES cells, as detected by PCR for the *lacZ* gene. Therefore, every rescued *Foxd3*–/– embryo contained wild-type cells. *Foxd3*–/– chimeras might be underrepresented for two reasons. First, most chimeras were recovered at 9.5 dpc, and it is possible that nonrescued embryos would be resorbed by that time. Second, the yolk sac endoderm was successfully separated from the extraembryonic mesoderm in most, but not all, cases, so it is possible that some *Foxd3*–/– embryos were incorrectly scored as +/–. Histological analysis of the chimera shown in Figure 5a demonstrated that after rescue with wild-type cells, *Foxd3*–/– cells can differentiate into axial and paraxial mesoderm, as well as neural crest (Fig. 5c,d; data not shown). This result indicates that injected wild-type ES cells act non-cell autonomously to rescue development of *Foxd3*–/– embryos.

Foxd3 *loss of function causes a loss of progenitor cells in the epiblast*

To assess the developmental potential and proliferative capacity of *Foxd3*–/– cells, we attempted to generate teratocarcinomas by placing the embryonic portion of a 6.5-dpc embryo under the kidney capsule of a histocompatible mouse. *Foxd3*+/+ and +/– teratocarcinomas contained differentiated tissues representing all germ layers including muscle, adipose tissue, cartilage, neuroepithelial tissue, gut-like tubes, and dermoid cysts (Fig. 5e; data not shown). However, no tumors were obtained from 11 *Foxd3*–/– embryos transplanted, even when only the extreme distal tip was transplanted in an effort to avoid inhibition of teratoma production by extraembryonic ectoderm. Figure 5f shows the site of a *Foxd3*–/– transplant 8 wk after surgery as evidence that the *Foxd3*–/– cells failed to grow or differentiate. These data support the idea that the pluripotent cells of the epiblast are lost without *Foxd3* function.

Foxd3 *is required for ES cell derivation*

To assess the requirement for *Foxd3* in another pluripotent progenitor cell type, we attempted to generate *Foxd3*–/– ES cells by two independent methods. First, we tried to derive *Foxd3*–/– ES cells from blastocysts;

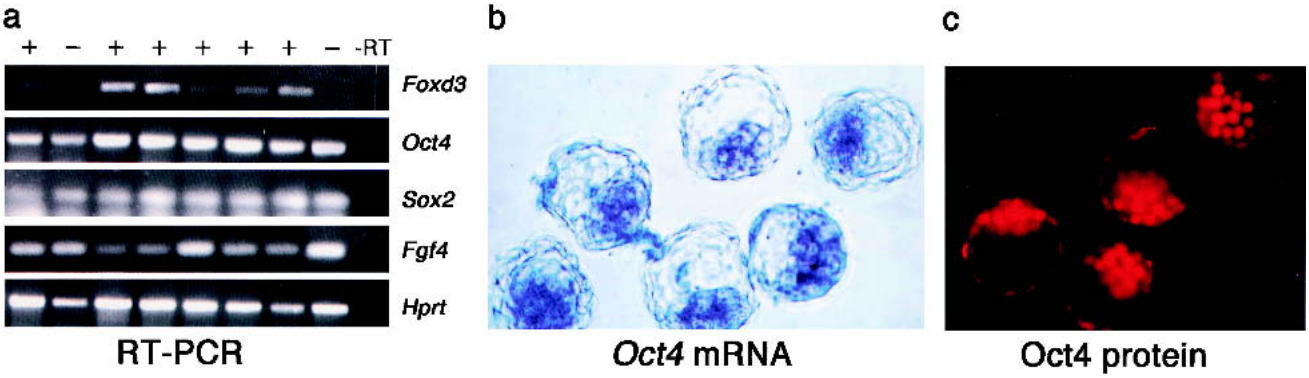

Figure 4. Expression of *Oct4*, *Sox2*, and *Fgf4* is maintained in *Foxd3*–/– blastocysts. (*a*) RT-PCR from single blastocysts shows that *Oct4*, *Sox2*, and *Fgf4* mRNAs are expressed in *Foxd3*–/– blastocysts. Lanes *2* and *8* are from *Foxd3*–/– embryos, and all other lanes are from either *Foxd3*+/+ or +/– blastocysts. (*b*) Whole mount in situ hybridization for *Oct4* mRNA demonstrated that blastocysts from intercrosses (18 of 18 total) have *Oct4* mRNA localized to the ICM. (*c*) Immunofluorescent detection of Oct4 protein in blastocysts from an intercross show that all blastocysts (23 of 23 total) have robust nuclear staining of Oct4 protein.

Foxd3+/– mice were backcrossed onto a 129SvEvTac genetic background for five generations, and 153 blastocysts from intercross matings were cultured to derive ES cell lines using standard methods (Hogan et al. 1994). Despite obtaining 6 *Foxd3*+/+ and 10 *Foxd3*+/– cell lines, no *Foxd3*–/– ES cell lines were established.

Next, targeting of the wild-type allele in *Foxd3*+/– ES cells was attempted. The *neo* cassette was removed from the targeted locus by electroporation with a plasmid encoding Cre recombinase. Colonies were picked at random, expanded, and tested by Southern blot for the presence of the *neo* cassette. To verify removal of the *neo* cassette, cell lines were also cultured in the presence of G418, and it was confirmed that no cells could survive this selection. The original targeting vector, shown in Figure 1c, was then reelectroporated into $Foxd3^{Ires\text{-}Gfp\Delta neo}$+/– cells, and cells were selected in G418. In several independent experiments, a total of 147 G418-resistant *Foxd3*+/– cell lines were obtained, indicating that the null allele had been retargeted in those cells. However, no *Foxd3*–/– ES cell lines were obtained in these experiments, suggesting that *Foxd3*–/– ES cells do not survive in culture. In addition, electroporated cells were grown in the presence of 12.5ng/mL recombinant Fgf4, similar to experiments that successfully generated *Fgf4*–/– ES cells (Wilder et al. 1997). In multiple experiments, 146 G418-resistant *Foxd3*+/– cell lines were obtained, but no *Foxd3*–/– cell lines were identified. Taken together, these results suggest that *Foxd3* function is essential for establishing pluripotent ES cells in vitro.

Foxd3 *is required for maintenance of the inner cell mass*

The inability to derive *Foxd3*–/– ES cells from blastocysts prompted an examination of the effect of prolonged in vitro culture on *Foxd3*–/– blastocysts. Blastocysts isolated from *Foxd3*+/– intercrosses were cultured in ES cell medium containing LIF. In culture, all blastocysts attached, hatched from their surrounding zona pellucida, and trophectoderm cells spread around a central ICM. For the initial 3–5 days of in vitro culture, *Foxd3*–/– outgrowths were indistinguishable from *Foxd3*+/+ or +/– (Fig. 6a). However, during days 5–9 in culture, the ICM of the *Foxd3*–/– embryos failed to expand, and trophoblast giant cells (arrows in Fig. 6a,b) underwent programmed cell death, as revealed by TUNEL staining (Fig. 6c). At the same time, *Foxd3*+/+ and +/– ICMs continued to expand and grow. Outgrowths were genotyped by PCR, and no outgrowths with a robust ICM were *Foxd3*–/–. In total, 16 of 69 (24%) blastocysts failed to survive in vitro. To test the possibility that a lack of Fgf4 from the ICM might be responsible for this phenotype, the culture medium was supplemented with 100ng/mL recombinant Fgf4 and 1μg/mL heparin. However, no difference was observed in the Fgf4-treated outgrowth cultures; 5 of 20 Fgf4-treated outgrowths failed to survive and expand an ICM, suggesting that Fgf4 cannot rescue the proliferation defect in *Foxd3*–/– ICM cells.

Discussion

The results presented here throw new light on the genetic regulation of early mouse development. The preimplantation mouse embryo contains only 20–40 cells in the ICM, and this pool of progenitor cells must proliferate and respond accurately to secreted signals to differentiate and undergo morphogenesis, forming both the embryo proper and supporting extraembryonic tissues. It is essential that the progenitor cells maintain their pluripotency throughout these critical stages and expand their cell numbers appropriately. We have now demonstrated that the winged helix gene *Foxd3* plays a role in maintaining the epiblast and its derivatives and in establishing pluripotent ES cell lines.

Foxd3–/– embryos die around the time of gastrulation with a loss of epiblast and a concomitant expansion of the proximal extraembryonic ectoderm and endoderm.

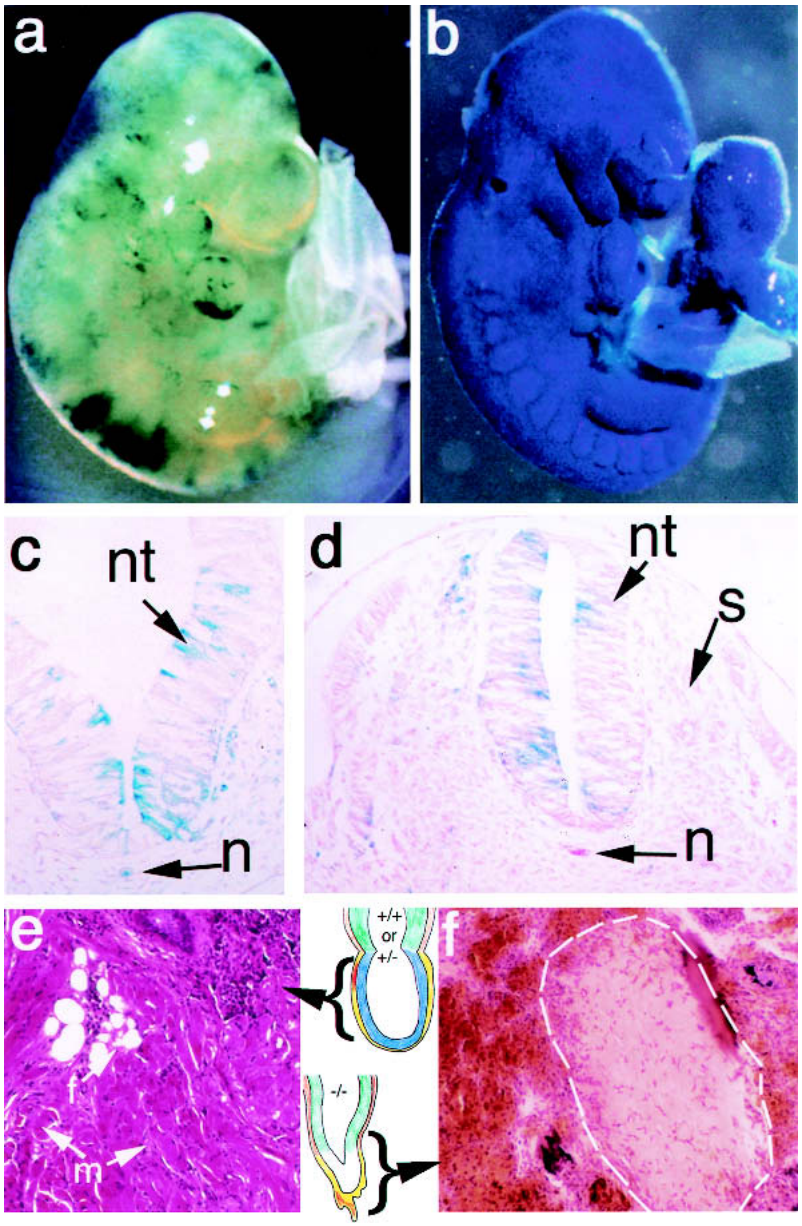

Figure 5. Developmental and proliferative potential of *Foxd3*–/– embryos. (*a*,*b*) Two examples of chimeras obtained by injecting *Rosa26.1* ES cells into blastocysts from *Foxd3*+/– intercrosses. Extraembryonic endoderm was used to genotype the recipient blastocysts, and chimeras were stained with Xgal to detect the wild-type *Rosa26.1* cells. Wild-type ES cells rescued the mutant phenotype completely, indicating that *Foxd3* function is required in the epiblast and that a low contribution of wild-type cells is sufficient to rescue the epiblast defect. (*c*,*d*) Histological analysis of chimera shown in panel *a* shows that *Foxd3*–/– cells (not stained blue) can differentiate into notochord, somites, and neural tube. (*e*,*f*) Teratocarcinomas derived from *Foxd3*+/– (*e*) and –/– (*f*) embryos. The portion of the embryo transferred is diagrammed. The heterozygous tumor extends far beyond the field shown and contains tissues derived from all three germ layers, including fat (f), skeletal muscle (m), neural tissue, and cartilage (data not shown). The *Foxd3*–/– embryonic cells do not produce any differentiated cell types and do not proliferate. The entire extent of the *Foxd3*–/– transplanted embryonic tissue is outlined with a white dashed line, and panels *e* and *f* are shown at the same magnification. Coagulated blood (orange) and calcium deposits (blue) are results of the surgery. f, fat; m, muscle; n, notochord; nt, neural tube; s, somite.

Our results suggest that the cells of the *Foxd3*–/– ICM and/or epiblast die. This conclusion is supported by in vitro blastocyst culture in which *Foxd3*–/– cells fail to proliferate and expand a normal ICM outgrowth. Mammalian embryogenesis requires a fine balance of extraembryonic and embryonic tissues. For example, when distal epiblast cells are transplanted to a proximal position adjacent to the extraembryonic ectoderm, these cells adopt a proximal cell fate, suggesting that signals from extraembryonic tissues influence the fate of epiblast cells (Tam and Zhou 1996). Thus far, only Bmp4 has been identified as an instructive signal produced by extraembryonic ectoderm; Bmp4 secretion from the extraembryonic ectoderm patterns the proximal portion of the epiblast and is required for generating extraembryonic mesoderm and precursors of primordial germ cells (Lawson et al. 1999). Such morphogenetic signaling occurs in both directions, and it has been suggested that Fgf4 secreted by the ICM and epiblast plays a role in patterning the extraembryonic ectodermal and trophoblast lineages (Tanaka et al. 1998). The apparent overgrowth of the extraembryonic ectoderm and loss of epiblast in *Foxd3*–/– embryos suggest that this balance is disrupted.

The chimeric rescue experiments show that wild-type ES cells completely rescue *Foxd3*–/– embryonic ectoderm through 9.5 dpc, indicating that Foxd3 function is required in the epiblast. In rescued mutants, *Foxd3*–/– cells can give rise to many different tissues and cell types, including mesoderm and neural crest. These re-

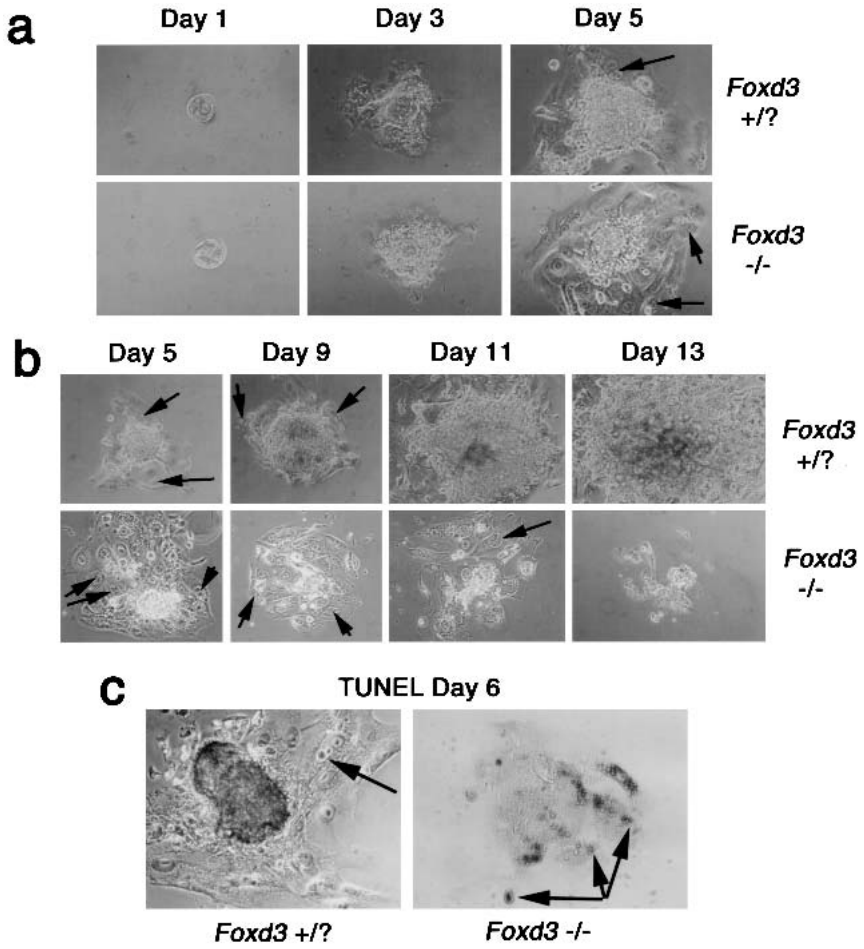

Figure 6. Developmental potential of the *Foxd3*–/– ICM in vitro. (*a*) Blastocysts were harvested from *Foxd3*+/– intercrosses and placed on gelatin-coated dishes in ES cell medium. Blastocysts attached and formed robust outgrowths within the first 3 d of culture. After prolonged culture (*b*), the *Foxd3*–/– cultures failed to maintain an ICM. Arrows in *a* and *b* indicate trophoblast giant cells. (*c*) TUNEL staining demonstrates that in *Foxd3*–/– outgrowths, attached cells were undergoing programmed cell death by day 6 in culture. Arrows in *c* point to TUNEL-positive cells.

sults show that Foxd3 functions in a non-cell autonomous manner and suggest that Foxd3 may be required for the regulation of a cell surface or secreted signaling molecule. Additional evidence indicates that cell-cell interactions may be critical for Foxd3 function. It has not been possible to generate *Foxd3*–/– ES cells by targeting the second allele of *Foxd3*+/– cells, even though the putative *Foxd3*–/– ES cells would be generated in the presence of *Foxd3*+/– ES cells. We would argue that secretion of a factor from wild-type ES cells is not sufficient to rescue *Foxd3*–/– cells because either cell-cell contact is required for such a rescue or the secreted factor must be present above a threshold level that is not attained under the in vitro culture conditions used. An alternative explanation is that wild-type cells must be present in a particular location, perhaps at the junction between the extraembryonic and embryonic regions of the embryo, in order for rescue to be achieved. Finally, a third possibility is that Foxd3 is necessary for the production or function of a factor required transiently to maintain the small population of pluripotent stem cells in the ICM or epiblast, but not for the subsequent differentiation of these lineages. A tissue-specific deletion of *Foxd3* will be required to address these issues.

It is known that the epiblast requires a sufficient number of cells to proceed past a critical stage in development (gastrulation), and one possibility is that following gastrulation, *Foxd3*–/– cells in chimeras can respond to other signals in the embryo and proliferate and/or differentiate accordingly. In support of this idea, mutations in the gene encoding the Bmpr/Alk3 receptor protein cause proliferation defects as early as 6.5 dpc with no production of embryonic mesoderm, although teratocarcinomas from *Bmpr*–/– cells, albeit smaller than wild type, are able to produce mesoderm (Mishina et al. 1995). The explanation for this difference is that the embryo requires a minimal number of epiblast cells to carry out the morphogenetic movements of gastrulation. Therefore, we would argue that in the *Foxd3* chimera rescue experiments, the presence of a sufficient number of wild-type cells allows progress through gastrulation.

The results show that the early preimplantation embryo is unaffected by the loss of Foxd3. The ICM of *Foxd3*–/– blastocysts appears morphologically indistinguishable from that of wild-type blastocysts. In addition, expression of mRNA for the transcription factors *Oct4* and *Sox2* and the secreted ligand *Ffg4* is detected in *Foxd3*–/– blastocysts, and Oct4 protein is localized to the nucleus. However, the ICM fails to properly expand in vitro, and cells from the ICM cannot respond to culture conditions designed to support ES cell derivation. *Oct4* expression is initiated but then downregulated

shortly after implantation in *Foxd3*–/– embryos (~5.5 dpc, Fig. 3a), and this may explain why *Foxd3*–/– embryos survive one day longer than *Oct4*–/– embryos. In agreement with this idea, *Oct4*–/– cells differentiate into trophoblast cells, and *Foxd3*–/– embryos have an expansion of the extraembryonic ectoderm, the trophoblast progenitor cells (Nichols et al. 1998). *Fgf4*–/– blastocysts also fail to elaborate an ICM in vitro, but they can be rescued by addition of soluble Fgf4 in the culture medium (Feldman et al. 1995). In contrast, *Foxd3*–/– ICMs are not rescued by addition of Fgf4, which is perhaps not surprising because mutant blastocysts are making *Fgf4* mRNA. This suggests that there may be a disruption in the ability of *Foxd3*–/– cells to receive or interpret the Fgf4 signal. It has been demonstrated in zebrafish that the *Oct4* homolog, *spiel-ohne-grenzen* (*spg*), is required for the competence of neurectoderm cells in the midhindbrain to respond to Fgf8 (Reim and Brand 2002). In addition, in vitro work has demonstrated that Foxd3 and Oct4 interact to regulate gene expression (Guo et al. 2002). Taken together, the analysis of the *Foxd3*–/– phenotype suggests that Foxd3 may be acting with Oct4 to confer responsiveness to Fgf4 signals on the ICM and early epiblast.

It has not been possible thus far to establish *Foxd3*–/– ES cell lines by several independent approaches. This is also the case for *Oct4*–/– ES cell lines, and careful titration of Oct4 levels in ES cells demonstrates that the absolute level of Oct4 protein is critical for the differentiation of specific lineages in the preimplantation embryo (Niwa et al. 1998). These results are in agreement with the model outlined above, placing Foxd3 function downstream of Oct4, required for the ability of cells to respond to Fgf4. A confounding observation is that *Fgf4*–/– ES cells have been isolated (Wilder et al. 1997). However, these cells were derived with soluble Fgf4 added to the medium, and it is not clear whether this is required for the establishment of such lines. This result raises an alternative explanation, placing Foxd3 in a parallel regulatory pathway, independent of Oct4, Sox2, and Fgf4, that is required for early embryogenesis and establishment of multipotent ES cells.

The winged helix gene *Foxd3* was first characterized by its expression in ES cells and multipotent cells of the neural crest. Ectopic expression of Foxd3 drives proliferation in cell culture and a change in fate of neural tube cells towards neural crest. Perhaps even more important for the potential control of stem cell fate, it was shown that continued expression of Foxd3 in migrating neural crest interferes with the subsequent differentiation of those cells (Dottori et al. 2001). The expression of Foxd3 in multipotent cells, the inhibition of differentiation by ectopic expression of Foxd3, and our studies of Foxd3 loss-of-function together suggest that Foxd3 may play a critical role in establishing or maintaining proliferating and self-renewing progenitor cell populations. Stem cells represent a unique tissue type with great potential for disease therapy. However, in order to take advantage of this important biological potential it is crucial that we understand the genetic pathways controlling the production, maintenance, and differentiation of these cells. Identification of key regulatory genes such as *Foxd3* is an important step in exploiting the potential for manipulation of multipotency in vitro and eventually in vivo for therapeutic goals.

Materials and methods

Generation of Foxd3+/– *mice and embryos*

The *Foxd3* locus was cloned by screening a 129SvEv mouse library (Research Genetics) using PCR and a probe from the cDNA (Labosky and Kaestner 1998). The targeting vector was constructed using a 4.0 kilobase (kb) *Sac*1 fragment as the 5′ homology arm and a 1.5kb *Xba*1 fragment as the 3′arm. A diptheria toxin (DTA) cassette was used to select against random integration, and positive selection was provided by the *neo* cassette, flanked by *loxP* sites for subsequent removal in culture. The *Foxd3* targeted allele has the entire coding region deleted as well as ~70 bp of the 3′ flanking DNA. Either a *Gfp* gene with an internal ribosomal entry site (Ires, for the $Foxd3^{Ires\text{-}Gfp}$ allele) or a *Gfp* cassette (for the $Foxd3^{Gfp}$ allele; Clontech) was inserted as diagrammed in Figure 1. With the *Ires-Gfp* cassette inserted, Gfp fluorescence was detected in *Foxd3*+/– ES cells, but this lineage reporter ($Foxd3^{Ires\text{-}Gfp}$) did not function in the embryo (data not shown). When a *Gfp* gene without an Ires was used, generating a fusion mRNA that included the *Foxd3* 5′UTR ($Foxd3^{Gfp}$), Gfp fluorescence was observed in the neural crest but never in embryos earlier than 8.5 dpc (data not shown).

TL1 ES cells were electroporated following standard protocols (Hogan et al. 1994), and 162 colonies were selected for resistance to G418 and screened by Southern blot as diagrammed in Figure 1. Thirty-six recombinants were obtained for a targeting frequency of 22%. Transient transfection with circularized *cre* plasmid was performed to remove the *neo* resistance cassette and generate the $Foxd3^{Ires\text{-}Gfp\Delta neo}$ allele. Three different cell lines carrying the $Foxd3^{Ires\text{-}Gfp}$ allele were injected into C57BL/6 blastocysts, and male chimeras were bred to either C57BL/6 or Black Swiss females. Two independent cell lines transmitted through the germ line, and because both showed the identical phenotype, studies were restricted to one cell line. Two independent cell lines carrying either the $Foxd3^{Ires\text{-}Gfp\Delta neo}$ or $Foxd3^{Gfp}$ allele were also used to generate those mouse lines.

Mice were genotyped either by Southern blot or by PCR with the following primers: wild-type allele, 5′-CGACGACGG GCTGGAGGAGAA-3′ and 5′-ATGAGCGCGATGTAAGGG TAG-3′; mutant allele, 5′-AAGGGCGAGGAGCTGTTCAC-3′ and 5′-TGCTGCTTCATGTGGTCGG-3′ (red boxes in Fig. 1c).

The mutation was maintained on several inbred and outbred genetic backgrounds (129SvEvTac, C57BL/6, Black Swiss, and CD-1) with no variation in phenotype. Removal of the *neo* cassette did not affect the phenotype (data not shown).

Chimeras

Chimeras were generated by injection of *Rosa26.1* ES cells into blastocysts isolated from *Foxd3*+/– intercrosses. Varying numbers (from 5 to 15) of ES cells were injected. Embryos were dissected at 8.5 and 9.5 dpc, and extraembryonic tissues were protease-treated to separate the extraembryonic mesoderm from the extraembryonic endoderm as described (Hogan et al. 1994). No chimeras were analyzed later than 9.5 dpc. DNA was extracted from extraembryonic endoderm to genotype the host

blastocysts retrospectively. Embryos were stained with Xgal using standard techniques (Hogan et al. 1994).

Histology and whole mount in situ hybridization

Histology was performed by standard approaches (Presnell and Schreibman 1997). BrdU incorporation was detected with an antibody from Zymed, and the TUNEL assays were performed as described (Conlon et al. 1995) with reagents from Roche Molecular Biochemicals and/or with the Apoptag kit from Intergen. Anti-phosphohistone H3 antibody was from Upstate Biotechnology, and Alexa Fluor® 488 goat-anti-rabbit secondary was from Molecular Probes. Oct4 antibody was from Becton Dickson. Whole mount in situ hybridization was performed using Costar 12-well inserts following standard protocols (Hogan et al. 1994). Digoxigenin-labeled RNA probes were prepared using reagents from Roche Molecular Biochemicals. To examine the expression of *Nodal*, mice carrying the *Foxd3* null allele and the *Nodal-lacZ* allele (Varlet et al. 1997) were generated. These double heterozygous mice were mated to *Foxd3*+/– mice, and embryos were stained with Xgal as described (Hogan et al. 1994) to reveal *Nodal* gene expression.

Teratocarcinomas

Teratocarcinomas were generated by transplanting a portion of a gastrula-stage embryo under the kidney capsule of a histocompatible adult mouse as described (Damjanov et al. 1987). For these experiments, transferred embryos were derived from crosses of *Foxd3*+/– mice on a C57BL/6 genetic background, and host mice were F1 hybrids from a [C57BL/6 × 129/SvEvTac] cross.

Derivation of ES cell lines

Blastocysts were harvested from 129SvEvTac (N5 or higher) *Foxd3*+/– intercrosses and placed onto irradiated STO feeder layers in complete ES medium: DMEM with 4500mg/L glucose and without pyruvate, 2mM glutamine, 0.1mM nonessential amino acids, 0.1mM β-mercaptoethanol (Sigma), 15% fetal calf serum (tested for ES cells, Summit Biotechnology), 1000U/mL LIF (ESGRO), and 50μg/mL gentamycin. Reagents were from GIBCO/Invitrogen except where indicated. After 4–5 d in culture when the ICMs have reached an appropriate size, they were manually isolated, trypsinized, and replated onto fresh STO feeder layers. After a few days in culture, ES cell clumps appeared and the line was then cultured routinely.

In vitro culture of blastocysts

Blastocysts were harvested from *Foxd3*+/– intercrosses and placed onto gelatin-coated dishes in ES cell medium. Fgf4 (Sigma) was added at 100ng/mL along with 1μg/mL heparin. Outgrowths were photographed every other day, and tissue was harvested and processed for RT-PCR as described below. Cultures were genotyped for *Foxd3* as indicated by *Foxd3* mRNA. Samples not demonstrating hypoxanthine phosphoribosyl transferase (*Hprt*) expression were discarded.

RT-PCR

RNA for RT-PCR was extracted using the Dynabead mRNA Direct kit (Dynal), and PCR was performed using the wild-type primers described above. To monitor the integrity of the cDNA produced by reverse transcription, RT-PCR for *Hprt* was performed with the following primers: 5′-TTGTTGGATTT GAAATTCCAGACAAG-3′ and 5′-GCATTTAAAAGGAACT GTTGACAACG-3′. The *Sox2*, *Oct4*, and *Fgf4* primers were as described (Nichols et al. 1998; Zappone et al. 2000).

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