Sox17 plays a substantial role in late-stage differentiation of the extraembryonic endoderm in vitro

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Summary

Sox17 is a Sry-related HMG-box transcription factor developmentally expressed in both the definitive endoderm and extraembryonic endoderm (ExE). Although *Sox17*$^{-/-}$ mouse embryos have a defective definitive gut endoderm, their developing ExE is morphologically intact. Here, we aimed to investigate the role of Sox17 in ExE development by using an in vitro differentiation system of embryonic stem cells (ESCs). Although forced Sox17 expression in ESCs did not affect ExE commitment, it facilitated the differentiation of ESC-derived primitive endoderm cells into visceral and parietal endoderm cells. This event was inhibited by the forced expression of Nanog, a negative regulator of differentiation of ESCs into the ExE. Although *Sox17*$^{-/-}$ ESCs could differentiate into primitive endoderm cells, further differentiation was severely impaired. These results indicate a substantial involvement of Sox17 in the late stage of ExE differentiation in vitro. Furthermore, the expression of Sox7 – another Sox factor, concomitantly expressed with Sox17 in the developing ExE – was suppressed during the in vitro differentiation of *Sox17*$^{-/-}$ ESCs, but it was maintained at a high level in the extraembryonic tissues of *Sox17*$^{-/-}$ embryos. These findings possibly explain the discrepancy between the ExE phenotype derived from *Sox17*$^{-/-}$ ESCs and that of *Sox17*$^{-/-}$ embryos.

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Introduction

Although the extraembryonic endoderm (ExE) does not contribute to the formation of all body cells, it plays crucial roles in mammalian development. By embryonic day (E) 4.5, a part of the inner cell mass of the mouse blastocyst differentiates into the primitive endoderm (PrE). PrE cells gradually cover the surface of the blastocoel cavity. By E5.5, they begin to differentiate further into two distinct cell lineages: the parietal endoderm (PE) and the visceral endoderm (VE) (Enders et al., 1978; Gardner, 1983). PE cells migrate and scatter on the inner surface of the parietal yolk sac. At E6.0, they begin to secrete extracellular matrix proteins, including type IV collagens and laminins. These proteins are later incorporated into Reichert's basement membrane, which forms a partition between the fetal and maternal environments (Gardner, 1983; Leivo et al., 1980). At the egg cylinder stage, the VE begins to cover the surface of the developing conceptus and the extraembryonic ectoderm (Gardner, 1983). The differentiated VE has functions related to embryo homeostasis, such as nutrient delivery, hematopoiesis and vasculogenesis (Bielinska, 1999). Moreover, the anterior part of the VE directly induces anterior patterning of the embryo during gastrulation (Beddington and Robertson, 1998).

A number of transcription factors, including Gata factors, Sox factors and hepatocyte nuclear factors (Hnfs), are expressed during the differentiation of embryonic stem cells (ESCs) or embryonal carcinoma cells into the ExE and its development. Loss-of-function studies have shown that most of these factors are essential for ExE development. *Gata6*-mutant embryos exhibit defective VE formation, resulting in embryonic lethality by E7.5 (Morrisey et al., 1998). Gata4-deficient ESCs are specifically defective in their ability to form the VE (Soudais et al., 1995). *Hnf3b* (*Foxa2*)-null mice have a broad spectrum of developmental abnormalities, including aberrant connections between the yolk sac and embryo proper (Farrington et al., 1997; Manova et al., 1998). Some of the developmental abnormalities are restored in *Foxa2*-null embryos that contain wild-type tetraploid yolk sac cells (Dufort et al., 1998), indicating that Foxa2 is indispensable for proper development of the ExE. Furthermore, a tetraploid complementation experiment demonstrated that Hnf4 is essential for proper functioning of the ExE (Duncan et al., 1997). By contrast, there exists a transcription factor that negatively regulates the differentiation of the inner cell mass or ESCs into the ExE lineage; this factor is known as Nanog, a homeobox-containing transcription factor that specifically

blocks the commitment of the inner cell mass and ESCs to the ExE lineage in vivo and in vitro (Chambers et al., 2003; Hamazaki et al., 2004; Mitsui et al., 2003).

The Sry-related high mobility group (HMG)-box-containing Sox transcription factors are involved in a wide range of developmental processes. Sox7 and Sox17 belong to the same subgroup F, and both are concomitantly expressed in the developing ExE (Kanai-Azuma et al., 2002). Sox17 is first detected in the ExE at E6.0 and it is gradually expressed in the entire ExE region by E6.5. Sox17 expression in the ExE is diminished by the early headfold stage. Sox7 expression in the ExE corresponds to Sox17 expression in the gastrula and neurula stages. Although Sox17 expression in the ExE coincides with the differentiation of ExE cells, *Sox17*$^{-/-}$ mice do not show morphological defects in the ExE (Kanai-Azuma et al., 2002). Sox17 is also expressed in the definitive endoderm, whereas Sox7 expression is restricted to the ExE. In contrast to the phenotype of *Sox17*-deficient ExE, the definitive gut endoderm of *Sox17*$^{-/-}$ embryos is severely impaired. This minimal effect of *Sox17* deficiency on ExE development might be attributed to functional compensation by Sox7. This speculation is supported by the fact that Sox7 plays a crucial role in the differentiation of F9 embryonal carcinoma cells into PE cells (Futaki et al., 2004). The involvement of Sox17 in the differentiation of the ExE has not yet been elucidated and is therefore worthy of further investigation.

When ESCs are allowed to aggregate in the absence of leukemia inhibitory factor (LIF), they form a structure known as the embryoid body (EB), which contains all three germ layers (Doetschman et al., 1985). The differentiated forms of ExE, namely VE and PE, arise from the outer layer of the EBs (Doetschman et al., 1985). However, when EBs are formed in the presence of LIF, the differentiation of ESCs into ExE lineages is arrested halfway. The intermediate ExE cells form the PrE, which consists of a thin single-cell layer on the surface of the EBs. The PrE cells express COUP-TF1 (Nr2f1), which is rapidly downregulated in the VE and PE cells, and Gata4, expression of which is maintained during further differentiation (Murray and Edgar, 2001a; Murray and Edgar, 2001b). These previous studies show that the process of differentiation of ESCs into the ExE can be divided at least into two distinct stages: in the early stage, ESCs differentiate into PrE cells, and in the late stage, PrE cells differentiate further into VE and PE cells. To address the role of Sox17 in the differentiation of the ExE in vitro, we examined whether forced Sox17 expression affects the differentiation process of ESCs into the ExE lineage. Using homozygous *Sox17*-mutant ESCs, we further investigated whether endogenous Sox17 is involved in the differentiation of ESCs towards the ExE.

Results

Generation of ESCs that conditionally express exogenous Sox17 under tetracycline control

To examine the precise effect of forced Sox17 expression on ESC differentiation, we generated ESC lines in which exogenous Sox17 expression was regulated by tetracycline (Tet) (Fig. 1A). The genomic ROSA26 promoter was used as a ubiquitous expression machinery (Zambrowicz et al., 1997) to achieve stable expression of a Tet-sensitive transactivator (tTA), and a tTA-responsive Sox17 expression cassette was flanked by the tTA transgene (Miyazaki et al., 2004). The regulated expression of exogenous Sox17 was readily detectable by the fluorescence of the concomitantly expressed enhanced green fluorescence protein (EGFP). We obtained two independent clones (clone 1 and clone 2) that were used for inducing both exogenous *Sox17* mRNA and a protein with no transgene expression in the presence of Tet (Fig. 1B,C). The minor band that is smaller than that of the typical Sox17 protein might correspond to a truncated form of Sox17 (Kanai et al., 1996).

The effect of forced Sox17 expression on ESC differentiation

To examine whether forced Sox17 expression induces

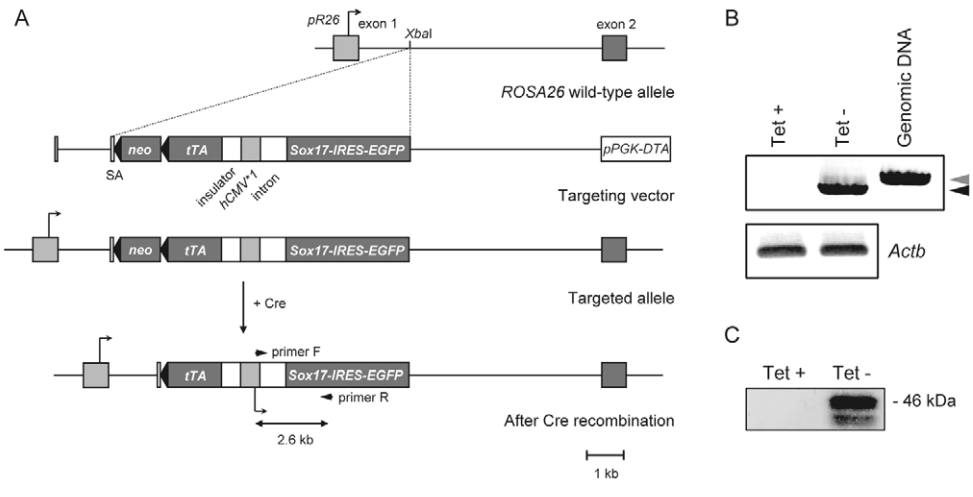

Fig. 1. Generation of ESCs with inducible exogenous Sox17 expression. (A) Schematic representation of the knock-in strategy of the inducible *Sox17* expression cassette into the *ROSA26* (*R26*) locus. The inducible *Sox17* expression cassette was inserted into the *Xba*I site in the *ROSA26* first intron. This cassette consisted of a splice acceptor (SA), floxed promoter-less neomycin-resistance gene (*neo*), tTA, insulator derived from the chicken β-globin locus, tTA-responsive human cytomegalovirus minimal promoter (hCMV*1), rabbit β-globin second intron, mouse full-length *Sox17* cDNA, internal ribosome entry site (IRES) and EGFP cDNA. Diphtheria toxin A (DTA) cDNA driven by the mouse phosphoglycerate kinase 1 promoter (pPGK) was placed at the 3′ end of the targeting construct for negative selection. After the targeting of the *ROSA26* locus, *neo* was excised by the transient expression of Cre recombinase. The locations of the PCR primers used to detect the exogenous *Sox17* transcript (primer F and primer R) are indicated. (B) RT-PCR analysis of exogenous Sox17 expression in ESCs grown in the presence or absence of Tet. The black arrowhead denotes the predicted size of the RT-PCR product (2.1 kb). Notice that this size is 0.5 kb less than that of the product derived from genomic DNA as a template (gray arrowhead). Actb is shown as the loading control. (C) Western blotting of the Sox17 protein. The cell lysates of ESCs grown with or without Tet were loaded.

differentiation of ESCs into the ExE lineage, we induced the *Sox17* transgene in ESCs grown in a maintenance culture with LIF and without Tet. The morphology of ESCs was not affected by forced Sox17 expression even after EGFP fluorescence reached a plateau (Fig. 2A). Transcripts of the PrE marker COUP-TF1 (Murray and Edgar, 2001a; Murray and Edgar, 2001b) and of transcription factors essential for ExE differentiation, including Sox7, Gata6, and Gata4, remained at low basal levels (Fig. 2B). Undifferentiated-state markers, including Nanog (Wang et al., 2003), Oct3/4 (Pou5f1) (Palmieri et al., 1994), Zfp42 (Rogers et al., 1991) and Sox2 (Yuan et al., 1995), were unaffected by forced Sox17 expression, which was quantified by real-time quantitative reverse transcriptase (RT)-PCR (qRT-PCR) (supplementary material Fig. S1). These results suggest that forced Sox17 expression did not induce differentiation of ESCs into the ExE lineage.

The surface layer of EBs in which differentiated VE and PE cells are present shows an irregular, cobblestone-like appearance (Soudais et al., 1995). After sufficient induction of Sox17, the ESCs were allowed to aggregate and were grown in a medium without LIF for 6 days, but the gross appearance of the EBs did not change, except for EGFP expression (Fig. 2C). Germ-layer markers, including Fgf5 (primitive ectoderm) (Haub and Goldfarb, 1991; Hebert et al., 1991), Isl1 (neurectoderm) (Ericson et al., 1992), T (brachyury; mesoderm) (Wilkinson et al., 1990), Wnt3 (mesoderm) (Liu et al., 1999), Gsc (mesendoderm) (Tada et al., 2005), Afp (VE) (Dziadek and Adamson, 1978) and Lamb1-1 (PE) (Senior et al., 1988), were expressed essentially at the same level as in the control EBs (Fig. 2D), suggesting that, even in the presence of forced exogenous Sox17 expression, the ESCs maintained their ability to differentiate into the three germ layers during EB formation.

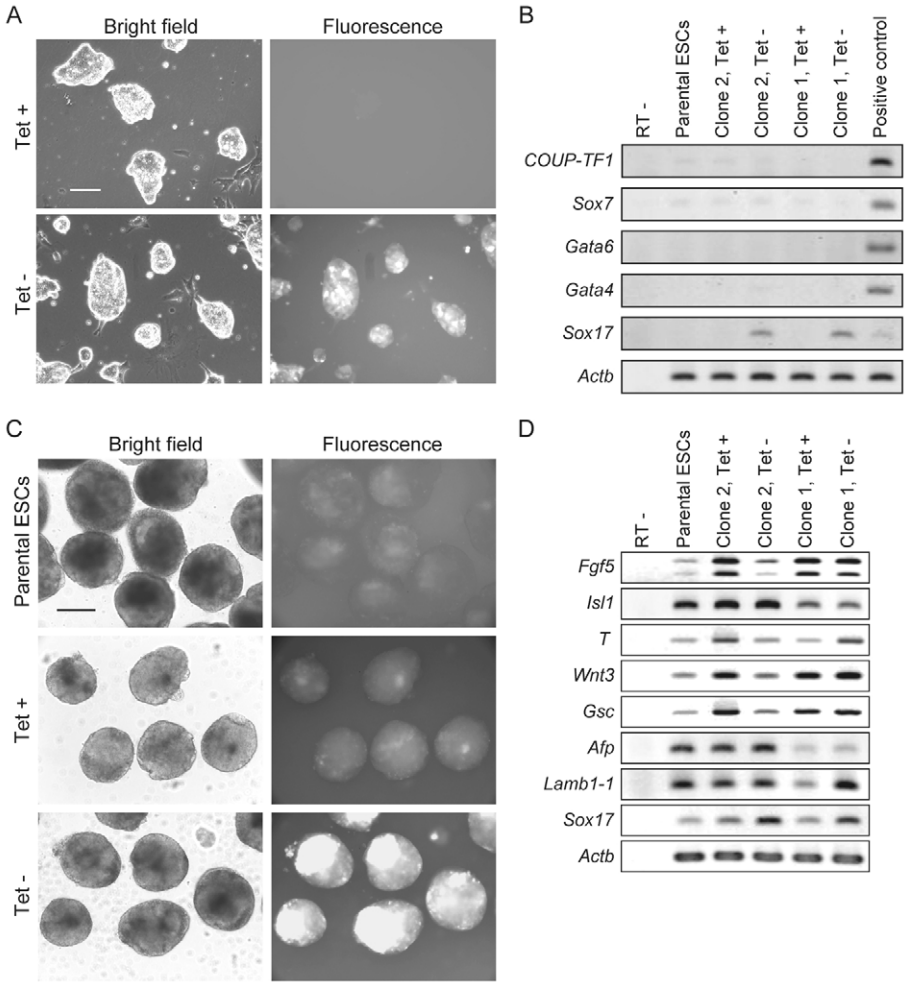

Fig. 2. Effect of forced Sox17 expression on ESC differentiation. (A) Morphology and EGFP fluorescence of ESCs grown with or without Tet. Bar, 100 μm. (B) RT-PCR analysis of ExE markers in the ESCs grown with or without Tet. cDNA derived from EBs of parental ESCs grown without LIF for various time periods was used as a positive control, and the corresponding RNA was used as the RT control. The *Sox17* primer set was designed to detect both endogenous and exogenous *Sox17*. (C) Morphology and EGFP fluorescence of EBs. ESCs grown with or without Tet were allowed to aggregate and were grown in LIF-deficient medium with the same Tet status for 6 days. Bar, 200 μm. (D) RT-PCR analysis of germ-layer markers in EBs grown with or without Tet in LIF-deficient medium. Two independent targeted clones were examined with similar results (A-D).

The impact of forced Sox17 expression on the morphology of EBs grown with LIF

When ESCs are allowed to aggregate in the presence of LIF, the surface layer of EBs differentiates into the PrE, but further differentiation into the VE and PE is inhibited (Murray and Edgar, 2001b). We applied this system to Sox17-inducible ESCs to examine whether forced Sox17 expression promotes the differentiation of the PrE into the VE and PE. First, we evaluated the effect of tetracycline on both the early and late stages of ExE differentiation during EB formation. After growing the parental ESC line EB3 with or without Tet for 4 days, the ESCs were allowed to aggregate and were grown with or without LIF for 6 days. Early ExE markers, including Gata6 and COUP-TF1, were expressed in EBs grown with LIF at the same level, independent of Tet treatment. Similarly, there were no differences in the expression of late ExE markers, including Ihh and Afp, in EBs grown without LIF (supplementary material Fig. S2). These results rule out the possibility that Tet affected the differentiation of ESCs into the ExE. Next, we determined whether forced Sox17 expression affected the late stage of ExE differentiation in vitro. After sufficient induction of Sox17, the ROSA26-targeted ESCs were allowed to aggregate and were grown with LIF for 10 days (EBs – Tet). Bright-field images demonstrated that EBs – Tet had irregular, cobblestone-like surfaces, whereas controls (EBs + Tet) displayed smooth, spherical surfaces (Fig. 3A,B), suggesting that forced Sox17 expression affects the surface structure of

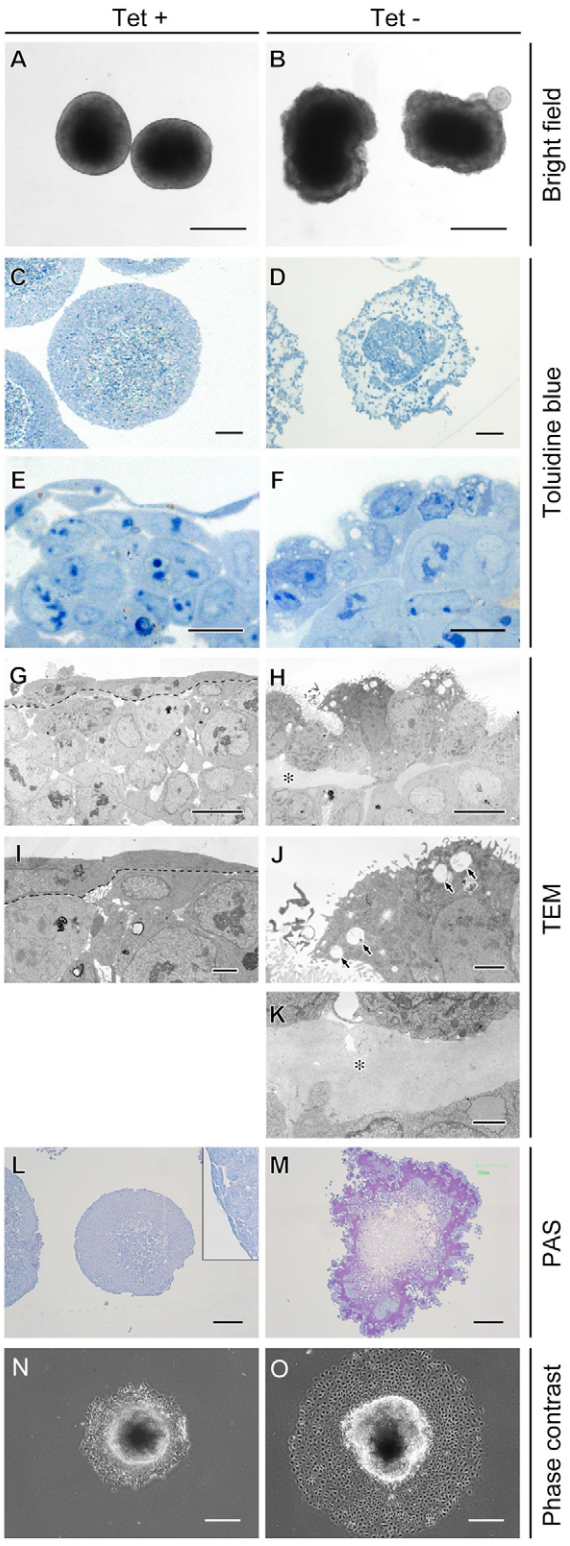

Fig. 3. Morphology of EBs grown with or without Tet in LIF-supplemented medium. After culture in LIF-supplemented medium without Tet for induction of exogenous Sox17, the ESCs were allowed to aggregate and were grown in the same medium for 10 days (Tet –). Control EBs were prepared using the same procedure, except that Tet was constantly supplemented to the medium (Tet +). (A,B) Bright-field images. Bars, 500 μm. (C-F) Toluidine-blue staining. Bars, 50 μm (C,D) and 10 μm (E,F). (G-K) TEM images. Boundaries between the PrE-like surface cells and the subjacent cells are shown as broken lines (G,I). The asterisks show the basement membrane-like structure (H,K). Arrows indicate vacuoles (J). Bars, 10 μm (G,H), 2 μm (I,K) and 1 μm (J). (L,M) PAS staining. The inset shows a magnified image (L). Bars, 100 μm. (N,O) Phase-contrast images of EBs grown in gelatin-coated tissue-culture dishes for 1 day. Bars, 300 μm. Two independent clones were examined with similar results (A-O).

EBs grown with LIF. Toluidine-blue staining confirmed the obvious structural difference depending upon the Tet status (Fig. 3C-F). The surface of EBs + Tet consisted of a thin single-cell layer, which is reminiscent of the PrE (Murray and Edgar, 2001b; Nadijcka and Hillman, 1974) (Fig. 3C,E). This structural property was confirmed by transmission electron micrography (TEM) (Fig. 3G,I). By contrast, the outer layer of EBs – Tet consisted of rounded cells with poor cell-cell contact, which is characteristic of PE cells (Casanova and Grabel, 1988; Enders et al., 1978), and consisted of cuboidal cells with an epithelium-like structure (Fig. 3D,F). Investigation of the subcellular structure of the cuboidal cells by TEM revealed numerous microvilli on the cells and revealed vacuoles on the apical side of the cytosol, which is characteristic of VE cells (Batten and Haar, 1979) (Fig. 3H,J). Moreover, a basement membrane-like structure was present just beneath the surface cell layer (Fig. 3K). Periodic acid-Schiff (PAS) staining further confirmed that the PAS-positive basement membrane was dramatically developed in EBs – Tet, whereas it was scarcely recognized in EBs + Tet (Fig. 3L,M). EBs at day 6 were allowed to attach onto gelatin-coated dishes to detect PE cells migrating away from the EBs. PE-like cells migrated away from EBs – Tet, but not from EBs + Tet (Fig. 3N,O). These morphological differences between the EBs suggest that, even in the presence of LIF, VE and PE cells existed in the outer layer of EBs when exogenous Sox17 expression was induced. There were no substantial differences between the two independent clones, indicating that the phenotype observed was not attributable to clonal variation.

To confirm and extend these morphological findings, we immunostained these EBs using antibodies against COUP-TF1, Gata4, Krt19 (PE marker) (Dufort et al., 1998) and Foxa2 (VE marker) (Kemler et al., 1981). In the outermost layer of EBs + Tet, COUP-TF1 and Gata4 were strongly expressed, and Krt19 and Foxa2 were expressed only in trace amounts (Fig. 4A-D, left), confirming the presence of PrE cells in the layer. However, in the external layer of EBs – Tet, COUP-TF1 expression was not evident, and Gata4, Krt19 and Foxa2 were strongly expressed, suggesting that VE and PE cells were present (Fig. 4A-D, right). These results regarding morphology and immunofluorescence indicate that, when forced Sox17 expression was induced in EBs grown with LIF, VE and PE cells were present instead of PrE cells.

The temporal expression pattern of ExE markers during EB differentiation that depends upon forced Sox17 expression

To investigate whether forced Sox17 expression facilitates the formation of the VE and PE, we analyzed the temporal pattern of gene expression associated with ExE differentiation during the culture of EBs with LIF. After sufficient induction of exogenous Sox17, ESCs were allowed to aggregate and were

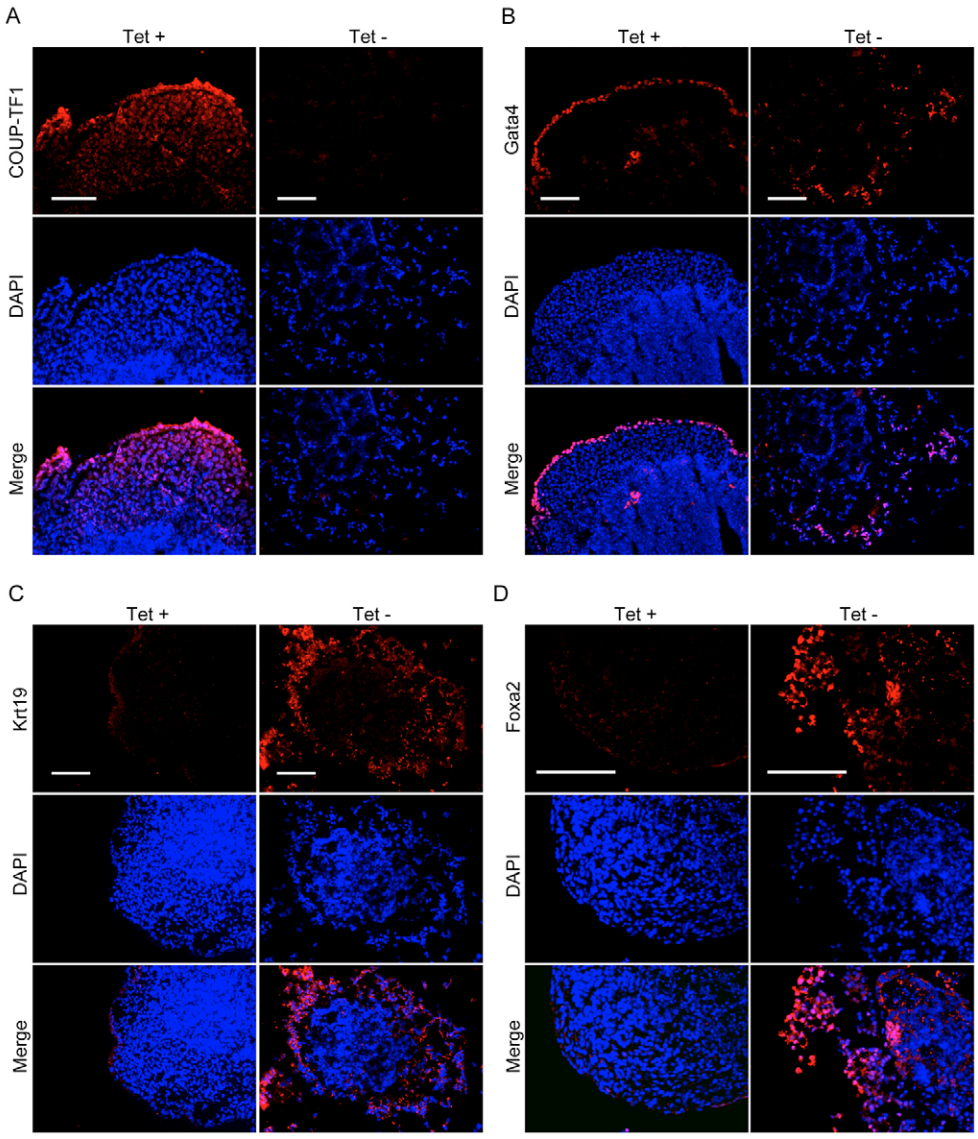

Fig. 4. Immunostaining of EBs with ExE markers. Anti-COUP-TF1 (A), anti-Gata4 (B), anti-Krt19 (C) and anti-Foxa2 (D) antibodies were used for immunostaining of EBs grown with (+) or without (–) Tet in LIF-supplemented medium. Two independent clones gave similar results. Bars, 100 μm.

grown with LIF for up to 6 days. For comparison purposes, the parental ESC line EB3 was allowed to aggregate and was grown in the presence or absence of LIF. cDNAs prepared at each time-point were subjected to qRT-PCR (Fig. 5). A *Sox17* primer pair was designed to detect both exogenous and endogenous transcripts. We confirmed sufficient induction of Sox17 in EBs – Tet. The VE markers Ihh (Becker et al., 1997) and Afp, and the PE markers Lamb1-1 and Sparc (Mason et al., 1986) were all upregulated to a considerably higher extent in EBs – Tet than in other control EBs. Transcription factors that regulate ExE differentiation, including Sox7, Gata6, Gata4, Foxa2, Hnf1b (Barbacci et al., 1999) and Hnf4 (Hnf4a) (Duncan et al., 1994), showed a similar expression profile as the VE and PE markers. Therefore, these results indicate that forced Sox17 expression facilitates the late-stage differentiation of ExE towards the VE and PE.

The impact of Nanog misexpression on ExE differentiation promoted by forced Sox17 expression

Nanog downregulation is obligatory for commitment of the inner cell mass and ESCs to the ExE lineage (Chambers et al., 2003; Hamazaki et al., 2004; Mitsui et al., 2003). One proposed function of Nanog is that it represses Gata6 (and thereby Gata4), inducing the commitment of ESCs to the ExE lineage (Fujikura et al., 2002). To investigate whether forced Sox17 expression can surpass the function of Nanog that prevents ESCs from differentiating into the ExE, we generated exogenous Sox17-inducible ESCs stably bearing a constitutive *Nanog* expression transgene. First, we confirmed that the exogenous Nanog inhibited the expression of early ExE markers, including COUP-TF1, Gata6 and Gata4, in EBs in which the *Nanog* transgene was present when they were grown in media without LIF and with Tet (not shown). After exogenous Sox17 was fully induced, the Nanog transfectants and control clones were allowed to aggregate and were grown with LIF for 10 days. In contrast to the control EBs (labeled as Tet –, Nanog – in Fig. 6), the surfaces of which showed an irregular, cobblestone-like appearance, the surfaces of EBs from Nanog transfectants (labeled as Tet –, Nanog +) were smooth and spherical (Fig. 6A). Uniform expression of the

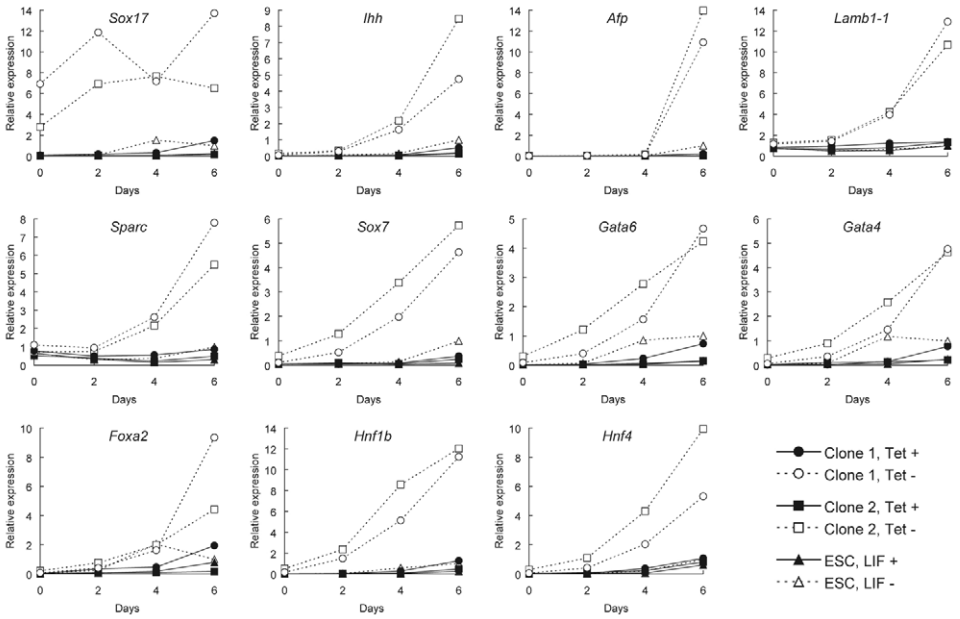

Fig. 5. The temporal expression pattern of ExE markers during EB differentiation. After culture in LIF-supplemented medium with or without Tet for several passages, ESCs with the inducible *Sox17* transgene were allowed to aggregate and were grown in the same medium for the indicated periods. Additionally, the parental ESCs were allowed to aggregate and were grown with or without LIF for the indicated periods. The expression levels relative to those in EBs derived from parental ESCs grown for 6 days are shown as the mean values of duplicate experiments. The *Sox17* primer set was designed to detect both endogenous and exogenous *Sox17*.

ROSA26-targeted transgene was confirmed by EGFP fluorescence in both EBs (Fig. 6B). Nanog misexpression in EBs derived from Nanog transfectants was confirmed by immunostaining with anti-Nanog antibodies (Fig. 6C). To investigate whether Nanog- and Sox17-overexpressing EBs contain ExE cells, we immunostained the EBs using antibodies against Gata4, an ExE marker. The result shows that Gata4-expressing ExE cells were not present in the Nanog-misexpressing EBs (Fig. 6D). Immunostaining of Krt19 revealed that Krt19-positive cells were not evident on the external surface of the EBs derived from Nanog transfectants (Fig. 6E). These results suggest that the effect of forced Sox17 expression on ExE differentiation cannot surpass the gatekeeping activity of Nanog.

We quantified the expression levels of transcription factors involved in ExE differentiation by qRT-PCR to examine whether these transcription factors were downregulated by Nanog misexpression (Fig. 6F). We confirmed that Nanog expression was approximately 1.7-fold higher in Nanog transfectants than in control EBs and that the Sox17 expression level was identical between the two groups. Ihh and Sparc levels were decreased in Nanog transfectants, consistent with the findings described above. The decreased expression of COUP-TF1 and Gata4 suggests that ExE differentiation was inhibited from the early stage by Nanog misexpression. Interestingly, Sox7 and Hnf1b expression was not reduced despite Nanog misexpression, suggesting a complex interaction between these transcription factors.

Effect of Sox17 deficiency on the in vitro differentiation of ESCs into ExE

We investigated the effect of Sox17 deficiency on ExE differentiation by using a *Sox17*$^{-/-}$ ESC line (Kanai-Azuma et al., 2002). Wild-type and *Sox17*$^{-/-}$ ESCs were allowed to aggregate and were grown for up to 12 days in a medium without LIF to drive ExE differentiation by the intrinsic machinery. The time-course of gene expression involved in ExE differentiation was estimated by qRT-PCR (Fig. 7A). We confirmed that Sox17 was not expressed in the *Sox17*$^{-/-}$ EBs. Marked decreases were observed in the expression levels of Ihh, Afp, Sparc and Sox7 in *Sox17*$^{-/-}$ EBs. Regarding Gata4, similar expression profiles were observed between wild-type and *Sox17*$^{-/-}$ EBs. Gata6 and COUP-TF1 expression in *Sox17*$^{-/-}$ EBs was enhanced, compared with wild type, after day 9. To explore the possibility that the differentiation of PrE cells into the VE and PE was impaired in *Sox17*$^{-/-}$ EBs, we investigated the morphology of these EBs by staining semi-thin sections with Toluidine blue and the expression of early ExE markers by immunostaining for Gata4 and COUP-TF1. A monolayer of flat cells, reminiscent of PrE cells, was present in both wild-type and *Sox17*$^{-/-}$ EBs grown for 3 days (Fig. 7B). Immunostaining of the EBs grown for 4 days revealed that the superficial cells were double-positive for Gata4 and COUP-TF1 (Fig. 7C), indicating that the formation of PrE cells occurred normally in these EBs. In EBs grown for 9 days, differentiated PE-like cells were present on the surface of wild-type EBs (Fig. 7B). By contrast, the superficial cells in *Sox17*$^{-/-}$ EBs still demonstrated a PrE-like thin single-cell layer (Fig. 7B). In wild-type EBs grown for 9 or 12 days, cells that were double-positive for COUP-TF1 and Gata4 decreased, indicating a decrease in PrE cells. By contrast, COUP-TF1 and Gata4 were mostly co-immunostained in *Sox17*$^{-/-}$ EBs grown for these durations, and the amount of co-immunostained cells was increased when compared with *Sox17*$^{-/-}$ EBs grown for 4 days (Fig. 7C). Although the results have been obtained using a single ESC line and a clonal artifact cannot be ruled out, they indicate that the differentiation of *Sox17*$^{-/-}$ PrE cells into VE and PE cells was impaired, and that PrE cells accumulated as a consequence.

Sox7 expression in *Sox17*$^{-/-}$ extraembryonic tissues

Our results concerning the in vitro differentiation of *Sox17*$^{-/-}$ ESCs is apparently inconsistent with the previous data obtained from *Sox17*$^{-/-}$ embryos, in which the formation of VE was normal (Kanai-Azuma et al., 2002). To address this discrepancy, we evaluated the expression of Sox7, which can

possibly compensate Sox17 deficiency, in the developing ExE. We prepared RNA samples from extraembryonic tissues of *Sox17* mutant mice at E7.75-E8.0 (corresponding to the early-headfold to early-somite stages), and estimated the expression of Sox7 and Sox17 in these samples relative to that in wild-type tissues by qRT-PCR. In contrast to the notable decrease in Sox7 expression in *Sox17*$^{-/-}$ EBs, Sox7 expression was maintained at a high level even in *Sox17*$^{-/-}$ extraembryonic tissues (Fig. 8).

Discussion

The present study illustrates that Sox17 is not involved in the early stage of ExE differentiation, and that Sox17 specifically participates in the late stage of differentiation of ExE towards VE and PE cells. Moreover, our results provide an insight into the functional relevance of Sox17 and other transcription factors that regulate ExE differentiation.

It has been shown that forced Sox17 expression does not induce differentiation of ESCs into the ExE lineage when ESCs are grown in culture dishes. Nanog, Oct3/4, Gata6 and Gata4 regulate the commitment of the inner cell mass or ESCs to the ExE lineage (Chambers et al., 2003; Mitsui et al., 2003; Morissey et al., 1998; Niwa et al., 2000; Palmieri et al., 1994; Soudais et al., 1995). When the expression levels of these genes are artificially changed in ESCs, they autonomously differentiate into the ExE lineages (Chambers et al., 2003; Fujikura et al., 2002; Mitsui et al., 2003; Niwa et al., 2000). Therefore, our result suggests that forced Sox17 expression does not induce ESC commitment. However, there remains a possibility that forced Sox17 expression induces only the commitment of ESCs to the ExE lineage, but not the differentiation of ESCs. This possibility is ruled out by the subsequent result suggesting that, even when exogenous Sox17 was expressed, the ESCs maintained their pluripotency. Consequently, these results indicate that forced Sox17 expression cannot induce the commitment of ESCs to the ExE lineage. Moreover, we have demonstrated that *Sox17*$^{-/-}$ ESCs could differentiate into PrE cells. This finding also shows that Sox17 is dispensable for the early stage of ExE differentiation in vitro. An in vivo study revealed that Sox17 expression in the ExE is induced after the PrE is produced from the inner cell mass, validating our idea in vivo (Kanai-Azuma et al., 2002). Taken together, these findings indicate that Sox17 is not involved in the early stage of ExE differentiation in vitro.

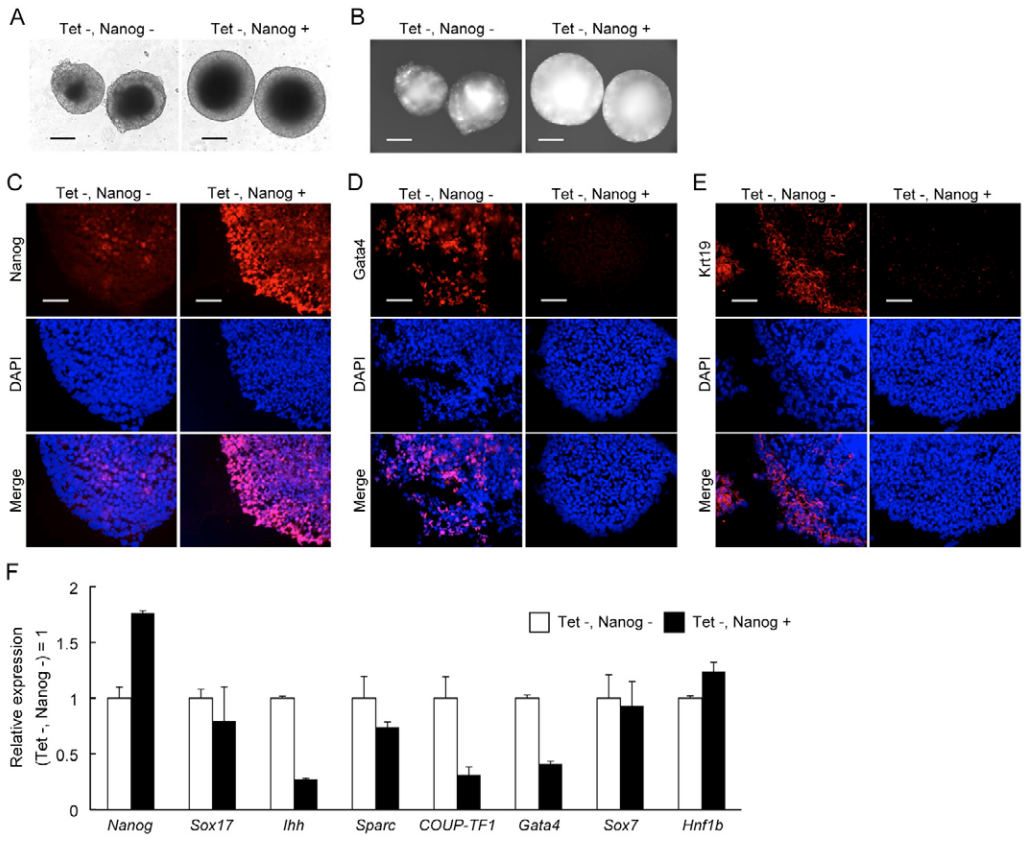

Fig. 6. The impact of Nanog misexpression on ExE differentiation promoted by forced Sox17 expression. ESCs bearing the inducible *Sox17* transgene were stably transfected with an empty vector or a constitutive *Nanog* expression vector. After being cultured in the absence of Tet in LIF-supplemented medium for *Sox17* induction, the ESCs were allowed to aggregate and were grown in the same medium for 10 days. (A,B) Bright-field (A) and fluorescence (B) images of EBs derived from a control (Tet –, Nanog –) clone and a Nanog-misexpressing (Tet –, Nanog +) clone. Bars, 200 μm. (C,D,E) Immunostaining of the control and Nanog-misexpressing EBs with anti-Nanog (C), anti-Gata4 (D) and anti-Krt19 (E) antibodies. Bars, 50 μm. Three independent Nanog clones and two independent control clones were examined with similar results (A-E). (F) Expression of ExE markers in the control (white bars) and Nanog-misexpressing (black bars) EBs. The expression levels relative to those in the control EBs, measured by qRT-PCR, are shown as the mean ± s.d. values of three independent experiments.

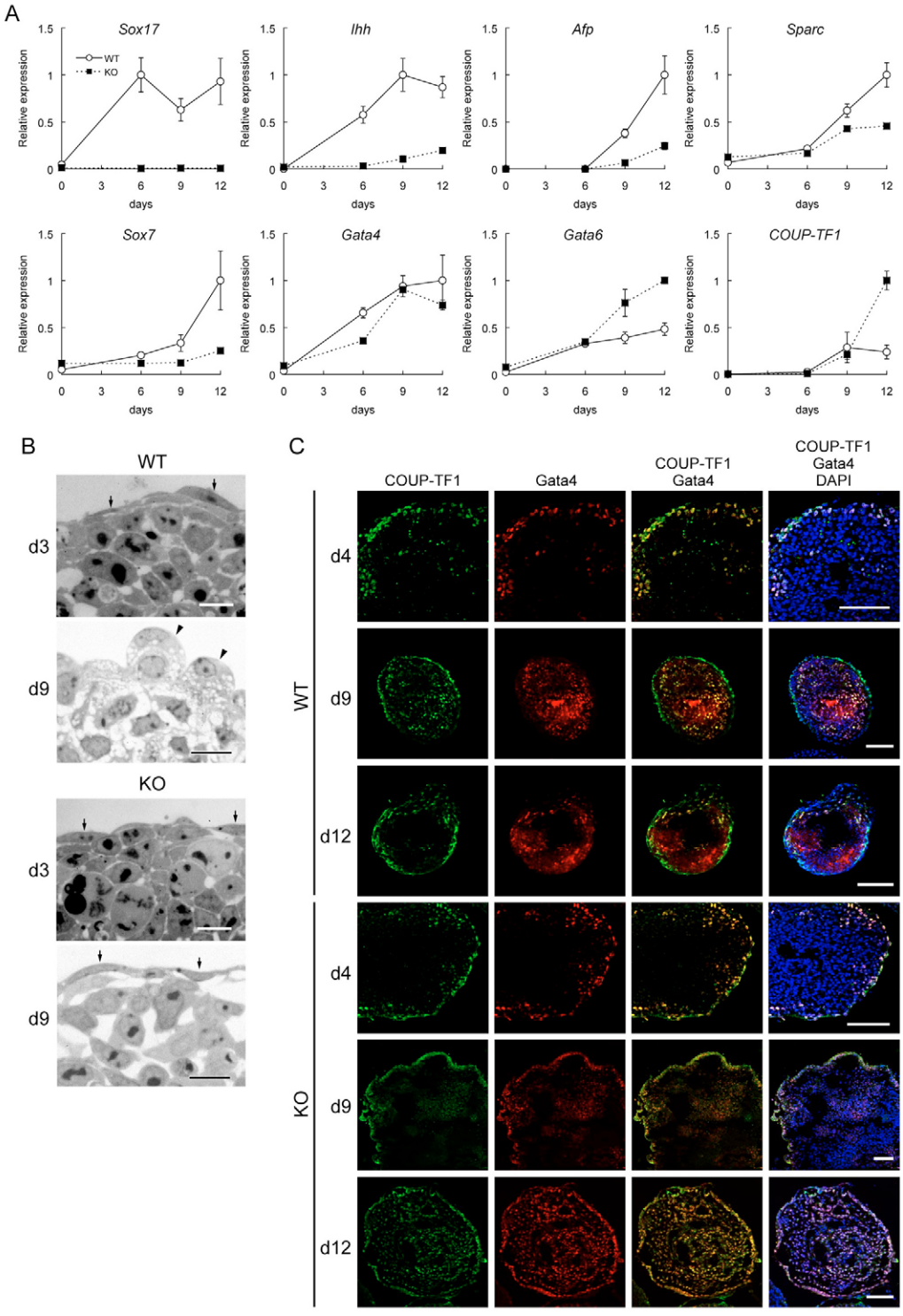

Fig. 7. Effect of Sox17 deficiency on ExE differentiation. (A) The temporal expression pattern of ExE markers in wild-type (WT) and *Sox17*$^{-/-}$ (KO) EBs. RNA from EBs grown without LIF for the indicated periods was subjected to qRT-PCR. The expression levels relative to the maximal levels are shown as the mean±s.d. values of two independent experiments. (B) Toluidine-blue staining of EBs grown without LIF for 3 or 9 days. Arrows, PrE-like cells; arrowheads, PE-like cells. Bars, 10 μm. (C) Immunostaining for COUP-TF1 and Gata4 in EBs grown for 4, 9 or 12 days. Bars, 50 μm.

During the differentiation of F9 embryonal carcinoma cells into PE cells, Sox7 is essential for induction of Gata4 and Gata6, whereas the Gata factors are essential for induction of Sox17 (Futaki et al., 2004), indicating a hierarchy from Sox7 to Gata4/Gata6 and from Gata4/Gata6 to Sox17. Furthermore, overexpression of Gata4 or Gata6 is sufficient to induce the differentiation of ESCs into VE and PE cells (Fujikura et al., 2002). Although Sox7 and Sox17 might have a redundant role in ExE development (discussed below), these findings, in theory, suggest that overexpression of the Gata factors can induce VE and PE cells even in the setting of Sox7 deficiency. Our results showed that, during EB differentiation in *Sox17*$^{-/-}$ ESCs, Sox7 was markedly downregulated, and Gata4 and Gata6 were expressed at similar or higher levels compared with those in wild-type EBs. Nevertheless, the differentiation of *Sox17*$^{-/-}$ ESCs into VE and PE cells was severely impaired.

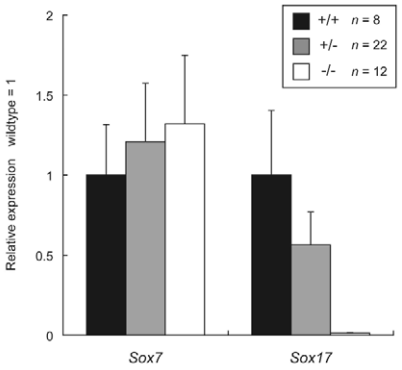

Fig. 8. Expression of Sox7 and Sox17 in *Sox17* mutant extraembryonic tissues at E7.75-E8.0. The amounts of *Sox7* and *Sox17* transcripts relative to those in wild-type (+/+) tissues, measured by qRT-PCR, are shown as the mean ± s.d.

Taken together, our results suggest that Sox17 is essential for the differentiation of ESCs into VE and PE cells in the setting of insufficient Sox7 expression. To formally prove the involvement of Sox17 in ExE differentiation, it is necessary to compare the ExE differentiation of *Sox7*$^{-/-}$ *Sox17*$^{-/-}$ ESCs with that of *Sox7*$^{-/-}$ *Sox17*$^{+/+}$ ESCs.

Our results shown here clearly indicate that forced Sox17 expression facilitates the late stage of ExE differentiation in vitro. By contrast, we have shown that the differentiation of PrE cells derived from *Sox17*$^{-/-}$ ESCs into VE and PE cells is severely impaired. These findings provide evidence that Sox17 is involved in the late-stage differentiation of the ExE in vitro. To our knowledge, there has been no report on transcription factors that specifically regulate the late stage of ExE differentiation. However, Sox7 appears to act as such a factor, because of the following reasons: forced Sox7 expression alone does not induce autonomous differentiation of F9 cells into PE cells, whereas *Sox7* knockdown suppresses the differentiation towards the PE lineage when F9 cells are treated with retinoic acid and dibutyryl cAMP (Futaki et al., 2004). Moreover, *Sox7* knockdown does not abolish the upregulation of COUP-TF2, which is expressed in the PrE (Murray and Edgar, 2001a). These findings are very similar to those observed in the case of Sox17. Therefore, we speculate that Sox7 is involved in the late stage of differentiation of the ExE into the PE, rather than in the commitment of the ESCs to the ExE lineage.

More recently, it was shown that cell lines of the ExE lineage, known as XEN cells, can be reproducibly established from the mouse blastocyst (Kunath et al., 2005). XEN cells show PE-like morphology and express transcription factors, including Gata4, Gata6, Sox7, Sox17, Hnf4 and Foxa2. XEN cells can differentiate into both VE and PE cells on changing the culture media or by injecting the cells into the blastocoel cavity. The ExE cells that were produced as a result of forced Sox17 expression had many properties in common with XEN cells, suggesting that XEN cells do exist to a considerable extent in the Sox17-expressing EBs grown in the presence of LIF. These findings raise a new question: how are the ExE cell types, including XEN, VE and PE cells, determined? Analyzing the transcriptional regulation in XEN cells will be a promising approach towards addressing this question.

Downregulation of Nanog and the subsequent upregulation of Gata factors are the central molecular processes in the early-stage differentiation of the ExE (Chambers et al., 2003; Fujikura et al., 2002; Hamazaki et al., 2004; Mitsui et al., 2003). Our results indicate that, when Nanog was misexpressed in EBs grown with LIF, forced Sox17 expression could not surpass the gatekeeping activity of Nanog that prevents ESCs from differentiating into the ExE lineage. This finding supports our conclusion that Sox17 is not involved in the early stage of ExE differentiation; that is, Sox17 can exert its function only after Nanog downregulation and upregulation of Gata factors in the ExE. Interestingly, when Sox17 was co-expressed with Nanog in EBs grown with LIF, the expression levels of Sox7 and Hnf1b were not reduced. Hnf1b, a transcription factor that regulates ExE development (Barbacci et al., 1999), is a putative transcriptional target of *Xenopus* Sox17 (Clements et al., 2003). Therefore, our results suggest that, even when Sox7, Sox17 and the target of Sox17 are present, these transcription factors cannot surpass the function of Nanog during ExE differentiation in vitro.

Sox17 and Sox7 might have a redundant function during ExE development and differentiation of F9 cells into the PE (Futaki et al., 2004; Kanai-Azuma et al., 2002). We hypothesized that the discrepancy between the ExE phenotypes of *Sox17*$^{-/-}$ embryos and EBs is due to the difference in the amount of Sox7 expression. The results shown in Fig. 7A and Fig. 8 clearly indicate that Sox7 expression was markedly reduced in *Sox17*$^{-/-}$ EBs, but not in *Sox17*$^{-/-}$ extraembryonic tissues. Therefore, these results suggest that Sox7 compensates for Sox17 deficiency in vivo and that the impaired ExE differentiation in *Sox17*$^{-/-}$ EBs is attributable to reduced Sox7 expression and Sox17 deficiency. Furthermore, these results indicate that the maintenance of Sox7 expression at a high level is not cell-autonomous. Futaki et al. have speculated that the stimulus mediated by the retinoic acid receptor RARb2 (Rarb) induces Sox7 expression in F9 cells (Futaki et al., 2004). It would be worthwhile to further investigate the regulatory signals that control Sox7 expression during mouse development.

In conclusion, the present study provides novel and important information on the functional significance of Sox17 in the regulation of ExE differentiation in vitro. We propose a model of the regulation of differentiation of ESCs into ExE cells by transcription factors (Fig. 9). Although Gata factors are essential for the early-stage differentiation of ESCs into the ExE lineage, Sox factors might act as the driving force for the late stage of ExE differentiation.

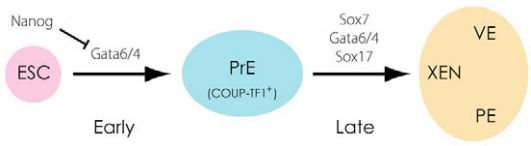

Fig. 9. Model of the regulation of differentiation of ESCs into ExE cells by transcription factors. The ExE differentiation process is divided into at least two stages. In the first stage, ESCs differentiate into COUP-TF1-positive PrE cells, and in the second stage, they differentiate further into XEN, VE and PE cells. We have demonstrated that Sox17 plays a substantial role in the late-stage differentiation.

Materials and Methods

DNA construct

The targeting vector for the ROSA26 locus was constructed as described previously (Miyazaki et al., 2004). Mouse *Sox17* cDNA was amplified from mouse testis cDNA by PCR. The primers used were 5′-TCTGGAGAGCCATGAGCAGC-3′ (forward) and 5′-GTCAAATGTCGGGGTAGTTG-3′ (reverse). Mouse *Nanog* cDNA was obtained from mouse MG1.19 ESCs (Gassmann et al., 1995) by PCR using the primers 5′-CCCTCGAGGGCCATCACACTGACATGAGTGTGGG-3′ (forward) and 5′-CCCTCGAGGGCGTAAGTCTCATATTTCACCTGGTGG-3′ (reverse). The PCR products were inserted into pBluescriptKS$^-$ (Stratagene) and confirmed by sequencing. *Sox17* cDNA was then inserted into the *Sfi*I site of the ROSA26 targeting vector. *Nanog* cDNA was inserted into the *Bst*XI site of the constitutive expression vector pCAG-IP (Niwa et al., 2002). The resulting vector was designated as pCAG-Nanog-IP.

ESC maintenance and transfection

The murine germline-competent ESC line EB3 (Niwa et al., 2002), a subline of E14tg2a (Hooper et al., 1987), and its derivatives were maintained in gelatin-coated dishes containing a Glasgow minimum essential medium (MEM)-based medium with 1000 U/ml LIF, as described previously (Moritoh et al., 2003). *Sox17*$^{-/-}$ and the parental W9.5 ESCs (Szabó and Mann, 1994) were maintained on primary mouse embryonic fibroblasts in Dulbecco's MEM supplemented with 15% fetal bovine serum, 2 mM L-glutamine, non-essential amino acids, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol and 1000 U/ml LIF. For transfection, 1×10^7 EB3 ESCs were electroporated with 50 μg linearized plasmid DNA at 0.8 kV and 3 μF by using Bio-Rad Gene Pulser. Selection was performed in the presence of 1 μg/ml Tet (Sigma), 150-200 μg/ml neomycin (Sigma) for ROSA26 targeting, or 1.5-2.0 μg/ml puromycin (Sigma) for pCAG-Nanog-IP or pCAG-IP transfection. The ROSA26-targeted clones were screened using a previously described method (Miyazaki et al., 2004). For Cre-mediated recombination, Cre expression plasmid DNA was transiently transfected as described previously (Taniguchi et al., 1998). Two independent lines (clone 1 and clone 2) of ESCs with an inducible *Sox17* transgene were maintained in the presence of 1 μg/ml Tet for suppressing the transgene. Three clones stably transfected with pCAG-Nanog-IP and two clones with pCAG-IP were generated from clone 1 as a representative clone because no substantial variations were observed in the ExE differentiation between the two lines, as described above. The ESCs with both the inducible *Sox17* transgene and pCAG-Nanog-IP or pCAG-IP were maintained in the presence of 1 μg/ml Tet and 1 μg/ml puromycin to allow efficient production of stable transfectants (Niwa et al., 2002). We confirmed the activity of the exogenous Nanog by culturing all clones in dishes containing LIF-deficient medium with Tet, as described previously (Mitsui et al., 2003).

ESC differentiation and RNA preparation

ESCs carrying an inducible *Sox17* cassette were passaged twice in the maintenance medium without Tet for sufficient induction of Sox17. After the induction of Sox17, the ESCs were trypsinized, washed and suspended in the same maintenance medium without Tet in the absence (Fig. 2C,D) or presence (Figs 3-6) of LIF at a concentration of 1000 cells/15 μl medium. Hanging culture was then achieved by spotting the cell suspension onto the lids of culture dishes (15 μl per drop). Hanging culture was performed for 2 days. Subsequently, the resultant EBs were transferred to bacterial dishes filled with the same medium and were grown for the indicated periods. The EBs were lysed directly with TRIzol reagent (Invitrogen) and RNA was prepared from the lysate according to the manufacturer's instructions. One RNA pool was derived from approximately 120 EBs. For control, Tet was constantly supplemented to the medium throughout the experiments and the same treatment method was used, except for the Tet status. *Sox17*$^{-/-}$ and wild-type W9.5 ESCs were trypsinized, passaged into gelatin-coated dishes, and incubated at 37°C for 30 minutes to allow feeder-cell attachment. The supernatant, which was ESC enriched, was then centrifuged. The cell pellet was washed and suspended in the Glasgow MEM-based medium without LIF. The procedure followed was the same as mentioned above.

cDNA preparation and RT-PCR analyses

cDNA synthesis and conventional RT-PCR analyses were performed as described previously (Fujikura et al., 2002). qRT-PCR analysis was carried out using SYBR Premix Ex Taq (TaKaRa). The reaction was performed with 1 μl cDNA per 50 μl reaction volume using ABI PRISM 7700 (Applied Biosystems) under the following thermal cycling conditions: 95°C for 10 seconds with no repeats, and 95°C for 5 seconds and 60°C for 30 seconds with 40 repeats. The relative quantity of the target transcript was estimated by the standard curve method using ABI PRISM Sequence Detection Systems 1.9.1 (Applied Biosystems), and the values were standardized by the relative expression values of Actb (β-actin). Most of the primer sequences used for RT-PCR analyses were designed according to our previous study (Fujikura et al., 2002) and the PrimerBank database (http://pga.mgh.harvard.edu/primerbank/) (Wang and Seed, 2003). The sequences are listed in supplementary material Table S1.

Western blotting

Samples from ESCs were subjected to western blotting as described previously (Fujikura et al., 2002). The antibodies used are listed in supplementary material Table S2.

Morphological analyses of ESCs and EBs

Bright-field and fluorescence images of the living cells were obtained using the Olympus IX70 fluorescence microscope. For toluidine-blue staining and TEM, the cultured EBs were fixed in 2.5% glutaraldehyde at 4°C for 12 hours. After post-fixation with 1% OsO_4, the specimens were dehydrated and embedded in Araldite M. Semi-thin sections (1 μm) were cut and stained with 1% toluidine blue (Kanai-Azuma et al., 2002). Ultra-thin sections were analyzed using the JEOL 1010 electron transmission microscope (Matsui et al., 2006). For PAS staining, the EBs were fixed in 10% buffered formalin and embedded in paraffin. The paraffin sections (3-4 μm) were stained with PAS according to the standard protocols. The images were obtained using Nikon COOLSCOPE.

Immunofluorescence staining

The EBs were embedded with an OCT compound (Sakura Finetech) and were frozen in liquid nitrogen. The samples were sliced at a thickness of 7 μm and were fixed with buffered 4% paraformaldehyde. Blocking was performed using 3% bovine serum albumin in PBS. The specimens were incubated overnight at 4°C with primary antibodies. After washing with 0.8% Tween 20-PBS, the specimens were incubated with Alexa-Fluor-488 or -568-conjugated antibodies (secondary antibody) at room temperature for 1 hour. After washing, they were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). Fluorescence images were obtained using the Olympus IX70 fluorescence microscope, and the raw images were processed using IPLab 3.5.2 (Scanalytics) for pseudocoloring and Adobe Photoshop Elements 2.0 for obtaining merged images. For co-immunostaining with anti-COUP-TF1 and anti-Gata4 antibodies, the specimens were sequentially probed with a mixture of these antibodies, the donkey anti-goat IgG antibody, and the goat anti-rabbit IgG antibody. Fluorescence images were obtained using the Radiance 2100 confocal microscope (Bio-Rad). The antibodies used are listed in supplementary material Table S2.

Mice

Sox17-mutant mice were generated as described previously (Kanai-Azuma et al., 2002). Extraembryonic tissues were isolated from E7.75-E8.0 embryos and subjected to RNA extraction as described earlier. Genomic DNA was extracted from the embryonic tissue and genotyping was performed by a PCR-based method (Kanai-Azuma et al., 2002). Animal experiments were conducted in accordance with the Guidelines for Animal Use and Experimentation formulated by the University of Tokyo.

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